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Anal

Effect of Dietary Fat Intake and Age on Polyunsaturated Fatty Acids in Human-Blood Serum

By V. S. PATIL and N. G. MAGAR
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(*Received* 28 *September* 1959)

During recent years many observers (Sinclair, 1956; Page, Stare, Corcoran, Pollack & Wilkinson, 1957; Mayer, Connell, DeWolf & Beveridge, 1954; Hardinge & Stare, 1954) have shown that animal and vegetable fats differ in their effect on lipids in blood serum of human beings. It has been suggested (Ahrens *et al.* 1957; Keys, Anderson & Grande, 1957; Malmros & Wigand, 1957; Bronte-Stewart, Antonis, Eales & Brock, 1956; Hegsted, Gotsis & Stare, 1957) that the effect of dietary fats upon lipids in serum depends upon the relative proportion of saturated to polyunsaturated fatty acids. Moreover, the concentration of polyunsaturated fatty acids in serum may have a bearing on the development of atherosclerosis (Hammond & Lundberg, 1955; Schrade, Biegler & Böhle, 1958; Antonis, 1958).

It is not yet certain whether linoleic acid and higher polyunsaturated fatty acids in serum decrease with age. In view of the important role played by diet as well as by age in the aetiology of atherosclerosis, an investigation of the nature of polyunsaturated fatty acids in human serum, especially among different age groups and groups receiving different diets and having different incidence of the diseases, will be of major importance. The present work was therefore undertaken to study (*a*) the effect of dietary fat intake on the polyunsaturated fatty acids in serum of normal human beings and (*b*) the concentration of polyunsaturated fatty acids in serum of human beings of age groups between 30 and 60 years and in atherosclerotic patients.

EXPERIMENTAL

The monthly food consumption of different families was recorded and from this the calorific and fat intakes/day/man were calculated (Patwardhan & Ranganathan, 1956). From the percentages of linoleic acid in hydrogenated fats (Patil & Magar, 1956), groundnut oil and sesame oil (Hilditch, Ichaporia & Jasperson, 1938) and in cereals, pulses, vegetables and foods of animal origin (Hilditch, 1956), the consumption of linoleic acid/day/man was calculated.

Clinical examination of the 'normal' subjects failed to show any disease. Atherosclerosis was diagnosed by clinical and electrocardiographic findings.

Analysis of polyunsaturated acids in human serum. About 3 ml. of serum from fasting human beings was analysed for total fatty acids according to the method of Wiese & Hansen (1953). Polyunsaturated fatty acids were estimated by alkali-isomerization–spectrophotometric methods (Herb & Riemenschneider, 1953). Calculations for the percentage of the individual polyunsaturated fatty acids were made according to Herb & Riemenschneider (1953), as modified by Hammond & Lundberg (1953), for the inclusion of hexaenoic acid. It was assumed that the pentaenoic acids consisted of 50% of docosapentaenoic acid and 50% of eicosapentaenoic acid. Values of t were calculated by Mather's (1949) method.

RESULTS AND DISCUSSION

Fat in the diet. The quantity of fat derived from cereals, vegetables and fruits consumed/day/man varied from 4·0 to 7·2 g., whereas that derived from fish, meat and eggs was 10–25 g. Hydrogenated vegetable fat and fat from milk consumed were 4–12 and 5–9 g. respectively, and about 21–25 g. of groundnut oil was consumed. About 36–42 g. of sesame oil was consumed by one vegetarian group.

Effect of linoleic acid on polyunsaturated fatty acids in human serum. Total fatty acids in serum of non-vegetarians of age groups 30–40, 40–50 and 50–60 years were significantly ($P \leqslant 0{\cdot}05$) higher than those of vegetarians on sesame oil (Tables 1–3). Total fatty acids in serum of vegetarians of age group 30–40 years were significantly lower than those of non-vegetarians (Table 1). Concentrations of tetraenoic acid in serum of vegetarians for age groups 30–40 and 50–60 years were also significantly high compared with those in non-vegetarians (Tables 1 and 3).

The concentrations of dienoic acid and tetraenoic acid in the serum of vegetarians on sesame oil in all age groups were significantly higher than those of the corresponding vegetarians and non-vegetarians (Tables 1–3). This may be due to the high intakes of linoleic acid (17·2–19·3 g.) in vegetarians who consumed sesame oil as compared with the intakes of linoleic acid in vegetarians (9·0–11·9 g.) and in non-vegetarians (8·4–9·5 g.). The concentration of trienoic acid in the serum of non-vegetarians was higher than that of the vegetarians on sesame oil for age group 30–40 years (Table 1). The value of pentaenoic acid for vegetarians on sesame oil was

Table 1. *Polyunsaturated fatty acids in the blood serum of human beings of age group* 30–40 *years*

Results are given ±S.D.

Group	No. of subjects	Daily fat intake (g.)	Daily linoleic acid intake (g.)	Total fatty acids (mg./100 ml.)	Polyenoic acids as percentage of total fatty acids				
					Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
I. Vegetarians	12	50·3±3·0	10·9±1·2	305±16·2	22·2±1·33	2·22±0·23	6·54±0·17	1·13±0·20	2·69±0·24
II. Non-vegetarians	7	58·1±3·5	9·54±1·0	328±12·4	21·7±0·83	2·21±0·32	6·26±0·26	1·19±0·28	2·60±0·22
III. Vegetarians on sesame oil	5	53·2±3·2	18·7±1·4	302±14·1	27·4±1·3	1·92±0·21	7·50±0·41	1·26±0·34	2·75±0·37
				t values for differences between groups					
I and II	—	—	—	3·06*	1·02	0·07	2·68*	0·51	0·77
II and III	—	—	—	3·41*	8·45*	0·61	5·57*	0·36	0·80
I and III	—	—	—	0·34	6·81	2·36*	6·41*	0·92	0·37

* Values significant at P 0·05.

Table 2. *Polyunsaturated fatty acids in blood serum of human beings of age group* 40–50 *years*

Results are given ±S.D.

Group	No. of subjects	Daily fat intake (g.)	Daily linoleic acid intake (g.)	Total fatty acids (mg./100 ml.)	Polyenoic acids as percentage of total fatty acids				
					Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
I. Vegetarians	8	46·5±3·4	9·67±0·9	326±23·5	21·4±1·73	2·04±0·22	5·82±0·53	1·03±0·12	2·44±0·32
II. Non-vegetarians	8	54·9±3·8	8·44±1·1	348±20·4	20·9±0·9	2·11±0·18	5·53±0·31	0·96±0·11	2·46±0·27
III. Vegetarians on sesame oil	57	57·5±2·9	19·3±1·5	315±18·4	26·3±1·91	2·0±0·31	7·52±0·84	1·20±0·21	2·72±0·40
				t values for differences between groups					
I and II	—	—	—	1·87	0·68	0·65	1·29	1·14	0·16
II and III	—	—	—	2·70*	6·29*	0·74	5·62*	2·48*	1·29
I and III	—	—	—	0·82	4·38*	0·65	4·19*	1·67	1·28

* Values significant at P 0·05.

Table 3. *Polyunsaturated fatty acids in blood serum of human beings of age group* 50–60 *years*

Results are given ±S.D.

Group	No. of subjects	Daily fat intake (g.)	Daily linoleic acid intake (g.)	Total fatty acids (mg./100 ml.)	Polyenoic acids as percentage of total fatty acids				
					Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
I. Vegetarians	8	42·0±2·8	9·0±1·1	352±32·7	20·6±0·94	2·27±0·15	5·59±0·13	0·82±0·12	2·18±0·22
II. Non-vegetarians	8	50·1±3·2	8·5±1·0	378±21·3	20·4±1·60	2·27±0·23	5·24±0·23	0·95±0·10	2·38±0·29
III. Vegetarians on sesame oil	5	47·7±2·5	17·2±1·3	334±27·4	25·6±1·1	2·12±0·18	6·42±0·41	1·00±0·16	2·40±0·45
				t values for differences between groups					
I and II	—	—	—	1·76	0·28	0·00	3·51*	2·77*	1·72
II and III	—	—	—	2·30*	5·87*	1·14	6·11*	0·60	0·10
I and III	—	—	—	0·94	7·98*	1·49	4·89*	2·13*	1·08

* Values significant at P 0·05.

Table 4. *Polyunsaturated fatty acids in human-blood serum*

Results are given ±S.D.

Age group	No. of subjects	Total fatty acids (mg./100 ml.)	Polyenoic acids as percentage of total fatty acids				
			Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
30–40	24	311 ±18·2	23·2 ±2·04	2·15 ±0·31	6·65 ±0·88	1·17 ±0·22	2·74 ±0·36
40–50	21	332 ±34·1	22·4 ±1·45	2·05 ±0·23	6·21 ±0·44	1·04 ±0·15	2·51 ±0·29
50–60	21	358 ±30·2	21·7 ±1·51	2·27 ±0·20	5·66 ±0·20	0·91 ±0·11	2·30 ±0·44
Atherosclerosis (50–60)	15	705 ±85·5	13·8 ±2·06	1·48 ±0·32	3·75 ±0·45	0·89 ±0·25	2·00 ±0·29
		t values for differences between groups					
30–40 and 40–50	—	2·56*	1·46	1·24	2·03*	2·23*	2·23*
40–50 and 50–60	—	2·55*	1·50	3·22*	5·00*	3·16*	1·77
50–60 and 30–40	—	6·23*	2·72*	1·48	4·99*	4·8*	3·54*
50–60 and atherosclerosis (50–60)	—	16·7*	12·9*	9·32*	16·7*	0·32	2·51*

* Values significant at P 0·05.

significantly higher than that for non-vegetarians for the age group 50–60 years (Table 3).

Effect of age on polyunsaturated fatty acids in human serum. As the age increased from 30 to 60 years the total fatty acids in the serum increased significantly and the concentrations of tetraenoic acid, pentaenoic acid and hexaenoic acid usually decreased significantly (Table 4).

The percentages of trienoic acid, pentaenoic acid and hexaenoic acid in serum of Indians (Table 4) were higher than those of blood-bank donors (1·59, 0·76 and 1·3 % respectively) reported by Hammond & Lundberg (1955). Total fatty acids (311–358 mg./100 ml.) in serum of Indians were low compared with those of Europeans (464 mg./100 ml.), reported by Antonis (1958), and of Guatemalans (394 mg./100 ml.) reported by Hammond & Lundberg (1955). However, the percentages of dienoic acid and tetraenoic acid (21·7–23·2 and 5·6–6·0 % respectively) in serum of Indians were similar to those (20·5–22·0 and 4·8–6·0 % respectively) reported by Antonis (1958) and Hammond & Lundberg (1955).

Polyunsaturated fatty acids in serum of atherosclerotic patients. The total of fatty acids in serum of atherosclerotic patients was significantly higher and the concentrations of dienoic acid, trienoic acid, tetraenoic acid and hexaenoic acid in serum were significantly lower than those of normal subjects of age group 50–60 years (Table 4). The results reported here are supported by the findings of Hammond & Lundberg (1955), Antonis (1958) and Schrade *et al.* (1958) in atherosclerotic patients. Hammond & Lundberg (1955) found 492 ± 38 mg. of total fatty acids/100 ml. of serum in atherosclerotic patients compared with the results (705 ± 85 mg./100 ml.) obtained in this experiment.

SUMMARY

1. The relative concentrations of dienoic acid and tetraenoic acid in the serum of Indians were proportional to the intake of linoleic acid from the diet.

2. Total fatty acids in serum increased, and the relative concentrations of dienoic acid, tetraenoic acid, pentaenoic acid and hexaenoic acid decreased, with age.

3. In atherosclerosis, total fatty acids in serum were prominently increased and the percentages of dienoic acid, trienoic acid, tetraenoic acid and hexaenoic acid were significantly lowered compared with those of normal subjects of the same age group.

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REFERENCES

Ahrens, E. H., jun., Hirsch, J., Insull, W., jun., Blomstrand, R., Tsaltas, T. T. & Peterson, M. L. (1957). *J. Amer. Oil Chem. Soc.* **34**, 563.

Antonis, A. (1958). In *Essential Fatty Acids*, p. 158. Ed. by Sinclair, H. M. London: Butterworths Scientific Publications.

Bronte-Stewart, B., Antonis, A., Eales, L. & Brock, J. F. (1956). *Lancet*, i, 521.

Hammond, E. G. & Lundberg, W. O. (1953). *J. Amer. Oil Chem. Soc.* **30**, 433.

Hammond, E. G. & Lundberg, W. O. (1955). *Arch. Biochem. Biophys.* **57**, 517.

Hardinge, M. G. & Stare, F. J. (1954). *Amer. J. clin. Nutr.* **2**, 83.

Hegsted, D. M., Gotsis, L. & Stare, F. J. (1957). *J. Nutr.* **63**, 377.
Herb, S. F. & Riemenschneider, R. W. (1953). *Analyt. Chem.* **25**, 953.
Hilditch, T. P. (1956). *Chemical Constitution of Natural Fats*, 3rd ed. London: Chapman and Hall Ltd.
Hilditch, T. P., Ichaporia, M. B. & Jasperson, H. (1938). *J. Soc. chem. Ind., Lond.*, **57**, 363.
Keys, A., Anderson, J. T. & Grande, F. (1957). *Lancet*, i, 66, 787.
Malmros, H. & Wigand, G. (1957). *Lancet*, ii, 1.
Mather, K. (1949). *Statistical Analysis in Biology*. London: Methuen and Co. Ltd.
Mayer, G. A., Connell, W. F., DeWolfe, M. S. & Beveridge, J. M. R. (1954). *Amer. J. clin. Nutr.* **2**, 316.
Page, I. H., Stare, F. J., Corcoran, A. C., Pollack, H. & Wilkinson, C. F. (1957). *Circulation*, **16**, 163.
Patil, V. S. & Magar, N. G. (1956). *J. sci. industr. Res.* **15B**, 650.
Patwardhan, V. N. & Ranganathan, S. (1956). *Health Bulletin*, no. 23, 5th ed. Nasik, India: Government of India Press.
Schrade, W., Biegler, R. & Böhle, E. (1958). *Dtsch. med. Wschr.* **83**, 1355, 1396.
Sinclair, H. M. (1956). *Lancet*, i, 381.
Wiese, H. F. & Hansen, A. E. (1953). *J. biol. Chem.* **202**, 417.

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Serum-Cholinesterase Activity in Health and in Liver Disease

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It has frequently been reported that abnormally low values of serum cholinesterase are found in the sera of patients suffering from liver disease. Although there is a considerable variation in the cholinesterase activity in normal sera (Callaway, Davies & Rutland, 1951), as assayed by the manometric method of Ammon (1933), abnormally low activities have been found in liver disease which are statistically significant (McArdle, 1940; Wilson, Calvert & Geoghegan, 1952). McArdle (1940) used acetylcholine as the substrate for the enzyme estimation and Wilson *et al.* (1952) used benzoylcholine.

Human-serum cholinesterase is now a fairly well described enzyme although its physiological role is not understood. The optimum pH for the hydrolysis of acetylcholine by the enzyme was found by Glick (1937) to lie between pH 8·4 and 8·5 at 25°. The substrate-specificity pattern of the enzyme has been extensively studied. Augustinsson (1949) found that butyrylcholine was the most rapidly hydrolysed substrate when a series of acyl choline esters were compared. Adams & Whittaker (1949) examined a series of alkyl esters and found that the maximum rates of hydrolysis were obtained with the esters of 3:3-dimethylbutanol, the carbon analogue of choline. Electrophoretic and fractionation studies reported by Surgenor *et al.* (1949): Surgenor & Ellis (1954); Kekwick, Mackay & Martin (1953) and Kekwick (1955) have shown that the enzyme is probably an α-globulin.

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In view of this fairly extensive knowledge of the nature of the enzyme in normal serum, and because the activity of the enzyme was known to be decreased in liver disease, it was thought that a comparison of the properties of the enzyme found in the serum of persons having liver disease with those of the enzyme of normal serum might reveal some differences.

MATERIALS AND METHODS

Enzyme assay. The enzyme assays were carried out in the Warburg apparatus at 37° in a medium containing 0·023M-sodium bicarbonate and 0·15M-sodium chloride at pH 7·4, according to the method of Ammon (1933). The gas phase was N_2+CO_2 (95:5). The substrates used were acetylcholine chloride and benzoylcholine chloride, both supplied by British Drug Houses Ltd., and the perchlorates of acetylcholine, propionylcholine and butyrylcholine, which were synthesized by the method of C. A. Lewis (personal communication). The concentration of acetylcholine, butyrylcholine and propionylcholine used was 0·06M and that of benzoylcholine was 0·12M. The concentrations were twice those found necessary by Mendel, Mundell & Rudney (1943) to give a maximal rate of hydrolysis of the substrate by the enzyme. These concentrations were also used in the study of the effect of temperature on the enzyme, in order to compensate for any change in the Michaelis constant with temperature. Estimations of enzyme activities at temperatures higher than 30° were obtained from measurements made during the first 30 min. after mixing serum and substrate; below this temperature it was necessary to prolong the period over which measurements were made owing to the lower reaction rates involved. The reactions proceeded linearly with time over the period of measurement in all cases, indicating that the enzyme was saturated with sub-

strate. The enzyme activity was measured as μl. of CO_2 evolved/hr./ml. of serum; in order to correct for any apparent change in the enzyme activity as a result of haemodilution, the enzyme activities of pathological sera have also been recorded as μl. of CO_2 evolved/hr./mg. of serum-protein nitrogen.

Estimation of protein nitrogen. Determinations of total nitrogen were carried out on serum by the micro-Kjeldahl procedure; to obtain protein nitrogen the blood urea was estimated and the corresponding nitrogen content was deducted from the total nitrogen found.

Determination of pH. The pH of the solutions used was measured by means of a glass electrode, 0·05 M-potassium hydrogen phthalate being used as standard at pH 4·0.

Sera studied. Normal sera were obtained from healthy persons aged between 18 and 50 years.

Pathological sera were obtained from patients suffering from liver disease classified in four groups. The group 'chronic liver damage' comprised patients with advanced hepatic insufficiency; a second group comprised patients whose conditions subsequently proved fatal, and in whom the diagnosis was confirmed at autopsy. The third group, those patients classified as having 'acute liver damage', were all in the acute phase of liver insufficiency, with fever, overt jaundice, a raised serum bilirubin, positive serum-thymol test and urobilinogen in the urine. Some of the patients whose sera were again assayed during the subsequent period of convalescence provided material for the fourth group, described as 'convalescent from acute liver damage'.

Some five sera from cases of chronic liver damage were selected for the specificity study; all of them had been observed for not less than 12 months and on four of them (1, 2, 3 and 4) the clinical diagnosis was confirmed at autopsy within 6 months of the analysis. The diagnosis of the remaining patient (5) was confirmed by biopsy.

Sera from six patients having acute liver damage were used for the specificity study: of these, three (6, 7 and 8) were examined within 14 days of the onset of jaundice, three (9, 10 and 11) were examined during a relapse occurring 4 weeks after the initial attack of infective hepatitis. Patient 10 had been confined to bed sick 1 year previously at a time when there was known to be an outbreak of hepatitis in his unit. Although at that time he had not become jaundiced it is possible that what was then regarded as 'abdominal 'flu' may have been a mild attack of infective hepatitis.

Sera were, if possible, assayed for activity as soon as they were taken, or were, if necessary, stored at 2° for a few days. It was found that cholinesterase activity of sera remained constant after storage for 300 days at 2° without any special precautions to maintain sterility.

RESULTS

Serum-cholinesterase activities in normal sera and sera from patients with liver damage

A comparison of serum-cholinesterase activities in liver disease with those found in normal persons was made initially with acetyl- and benzoyl-choline chloride as substrates, to provide a basis for a more extensive investigation of the behaviour of the enzyme in liver disease.

The results of the assays of enzyme activity with the chloride of acetyl- and benzoyl-choline are shown in Table 1. In sera from both of the groups of patients with chronic liver damage the mean activity against either substrate was markedly lower than that of normal sera. All the sera in these two groups had enzyme activities, which, when expressed in μl. of CO_2 evolved/hr./ml. of serum, were below the normal range, but when these activities were expressed in terms of μl. of CO_2 evolved/hr./mg. of protein nitrogen some of them fell just within the normal range. The range of activities found in the sera from the group of persons suffering from acute liver damage was different. Although the mean activity against both the substrates was below that found for normal sera, 12 of the 17 sera studied had activities within the normal range. It is possible that these levels actually represented lowered values for individuals normally having rather a high serum-cholinesterase activity, in view of the considerable range of levels found in normal sera.

Patients convalescent from acute liver damage had serum-cholinesterase activities which were all within the range for normal sera. In all the sera studied, both normal and pathological, the rate of hydrolysis of benzoylcholine was always between 35 and 40 % of that of acetylcholine.

Table 1. *Hydrolysis of acetylcholine and benzoylcholine by normal and pathological human sera*

		Acetylcholine				Benzoylcholine			
		CO_2 evolved (μl./hr./ml.)		CO_2 evolved (μl./mg. of N/hr.)		CO_2 evolved (μl./hr./ml.)		CO_2 evolved (μl./mg. of N/hr.)	
Group	No. in group	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Normal	20	5420	3180–7300	417	280–550	2050	1240–3100	186	109–250
Chronic liver damage	10	2150	1200–3050	208	136–352	815	500–1200	81	54–160
Chronic liver damage followed by death within 6 months	4	1647	500–3000	139	54–236	730	200–1500	54	25–91
Acute liver damage	17	4052	1130–6220	329	176–503	1612	515–3100	132	92–186
Convalescent from acute liver damage	5	5600	3800–7300	446	317–550	2073	1090–2180	165	91–212

Substrate-specificity pattern of serum cholinesterase

Sera obtained from five patients having chronic liver damage which subsequently proved fatal, and from six patients having acute liver damage, were selected for comparison with four normal sera with respect to their specificity towards aliphatic choline esters. The results of assays of the activities of sera against the perchlorates of acetyl-, propionyl- and butyryl-choline are shown in the form of a specificity pattern in Fig. 1. The behaviour of four normal sera towards the three substrates was similar to that described by Augustinsson (1949) for a purified preparation of serum cholinesterase, butyrylcholine being hydrolysed the most rapidly. The relative rates of hydrolysis did show slight individual variations.

Sera from patients having chronic liver damage behaved quite differently; propionylcholine was always hydrolysed most rapidly.

In the group of sera from acute liver damage only two out of six showed this abnormal preference for propionylcholine, but one of these sera had an activity against acetylcholine which was within the normal range. Of the remaining sera in the group two hydrolysed propionyl- and butyryl-choline at the same rate, and one serum had a normal specificity pattern despite a very low activity against acetylcholine.

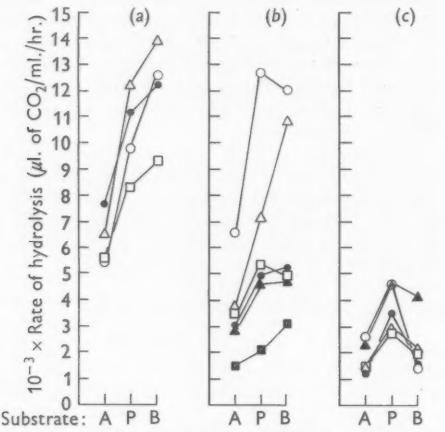

Fig. 1. Specificity of normal serum cholinesterase towards acetyl-, propionyl- and butyryl-choline. (*a*) Normal human serum: ○, NM; ●, RJM; □, CBC; △, RK. (*b*) Sera from patients (identified by numbers) with acute liver damage: ○, 6; △, 7; ▲, 8; □, 9; ● 10; ■, 11. (*c*) Sera from patient (identified by numbers) with chronic liver damage: ○, 1; △, 2; □, 3; ●, 4; ▲, 5. Substrates: A, acetylcholine; P, propionylcholine; B, butyrylcholine.

Effect of temperature and pH *on serum-cholinesterase activity*

The abnormal specificity pattern found for serum cholinesterase in some cases of liver damage suggested that in these pathological sera the enzyme might differ from the normal in some other characteristics. Consequently the variation of enzyme activity with temperature and pH in some pathological sera was compared with the normal in the hope that some further differences might be revealed.

Effect of temperature. Cholinesterase activities of sera towards acetyl- and benzoyl-choline chloride were measured over a range of temperatures from 2° to 60°; the reaction always proceeded linearly with time over the period of measurement; thus the enzyme always appeared saturated with the substrate. The rate of enzymic hydrolysis of acetylcholine was corrected for spontaneous hydrolysis, but this was negligible for benzoylcholine chloride.

The rates of hydrolysis of acetyl- and benzoylcholine by normal sera and by two sera from patients with chronic liver damage (both of which showed the abnormal specificity pattern) varied similarly with increase in temperature. The optimum temperature was, in all cases, 47° for both substrates. But a purified preparation of cholinesterase having a 20-fold increase in activity, which was obtained by the ether-fractionation technique of Kekwick & Mackay (1954) from pooled normal plasma (AP_1/P_3 of Table 2), had an optimum temperature at 45°. Since the enzyme reaction proceeded linearly with time in each measurement, no change of enzyme configuration could have occurred, and the optimum temperature is an indication of the thermal stability of the enzyme in the experimental environment.

As the enzyme was saturated with the substrate, the observed velocity (v) was maximal, and proportional to the velocity constant for the breakdown of the enzyme–substrate complex to give the reaction products.

Table 2. *Activation energies for the hydrolysis of choline esters by normal and pathological human sera*

	Activation energies (kcal./mole)	
	Acetylcholine	Benzoylcholine
(*a*) Normal sera		
NM	8·3	14·9
RK	9·2	12·5
RJM	9·3	13·2
Purified fraction AP_1/P_3	8·4	12·7
(*b*) Chronic liver damage		
Patient 13	9·4	—
Patient 1 (died)	7·8	12·3

Substitution of the results obtained for the velocity of the reaction at different temperatures in the Arrhenius equation (1889) shows that the activation energy for the hydrolysis of acetylcholine by normal serum varies from 8·3 to 9·3 kcal./mole and for the hydrolysis of benzoylcholine from 12·5 to 14·9 kcal./mole (Table 2). One serum from chronic liver damage (1) had a very slightly lower value for the activation energy of the hydrolysis of both acetyl- and benzoyl-choline, the other serum from chronic liver damage had a normal activation energy for the hydrolysis of acetylcholine. The purified preparation of serum-cholinesterase AP_1/P_3 had activation energies similar to normal serum. The activation energy for the hydrolysis of butyrylcholine by one normal serum (RK) was 8·5 kcal./mole.

All the values of the activation energies given were obtained from measurements made below 34°, because it was found that above this temperature the Arrhenius equation was no longer obeyed: the plot of log v against $1/T$ was no longer linear.

Effect of pH. The variation of serum-cholinesterase activity with pH at 37° was measured for both acetyl- and benzoyl-choline chloride over the narrow range from pH 7·1 to 7·8 permitted by the bicarbonate buffer system. The behaviour of two normal and two pathological sera was studied; one of the two pathological sera (4) was obtained from a patient having multiple myelomatosis but who showed clear evidence of extensive liver damage.

The results obtained are plotted in Fig. 2 (*a*); the optimum pH for the hydrolysis of acetylcholine is above pH 7·8 for normal serum, but for benzoylcholine it is about 7·4. The results obtained for pathological sera were similar but the variation in activity towards acetylcholine with pH was not so marked and the pH optimum for the hydrolysis of benzoylcholine by one serum (14) appears to be shifted to a more acid region.

It was thought that possibly an apparent shift in the optimum pH of the cholinesterase activity of serum might arise when the protein pattern of serum was grossly distorted, as in multiple myelomatosis. To test this, 5 ml. of a 4·2 % protein solution containing 90 % of γ-globulin prepared from normal pooled serum by the ether-fractionation technique of Kekwick & Mackay (1954) was added to 5 ml. of a normal serum (NM) containing 8·2 % of protein; this gave a solution containing 6·2 % of protein in which there was a high concentration of γ-globulin. The variation of cholinesterase activity with pH in the system is compared with that of the normal serum (NM) in Fig. 2 (*c*). The optimum pH for the hydrolysis of acetylcholine was shifted from above pH 7·8 to pH 7·4, but the optimum pH for the hydrolysis of benzoylcholine was not affected.

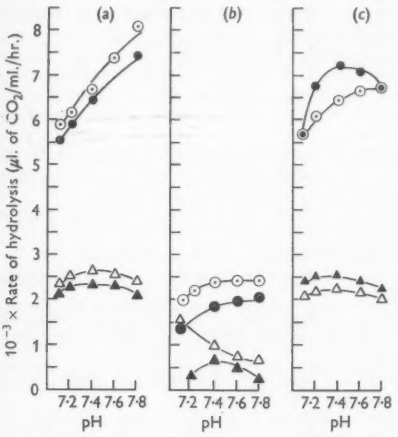

Fig. 2. Effect of pH on serum cholinesterase. (*a*) Normal serum: ○, NM hydrolysing acetylcholine (Ach); △, NM hydrolysing benzoylcholine (Bch); ●, RK hydrolysing Ach; ▲, RK hydrolysing Bch. (*b*) Serum from patients with chronic liver damage: ○, Serum 14 hydrolysing Ach; △, serum 14 hydrolysing Bch; ●, serum 4 hydrolysing Ach; ▲, serum 4 hydrolysing Bch. (*c*) Normal serum plus γ-globulin: ○, NM hydrolysing Ach; ●, NM + γ-globulin hydrolysing Ach; △, NM hydrolysing Bch; ▲, NM + γ-globulin hydrolysing Bch.

DISCUSSION

The values of the activation energy for the hydrolysis of acetylcholine by normal human serum are similar to those of Shukuya (1953), who also found that the Michaelis constant for this enzymic reaction is independent of temperature. This indicates that the same substrate concentration will give maximal hydrolysis at different temperatures, as was found.

The difference in the activation energies found for the hydrolysis of the aliphatic esters acetylcholine and butyrylcholine (about 8·5 kcal./mole) on the one hand and for benzoylcholine (13 kcal./mole) on the other, when considered with the observation that the optimum pH for the hydrolysis of acetylcholine is above pH 8·0, whereas that for benzoylcholine is about pH 7·4, suggests that the mechanism whereby benzoylcholine is hydrolysed is somewhat different from that of the aliphatic esters. For both normal and pathological sera the rate of hydrolysis of benzoylcholine was always a constant fraction of that of acetylcholine, which suggested that the same enzyme was always involved. Further evidence of the dissimilarity of the mechanisms involved comes from the observation of Augustinsson (1949), who has shown that

the hydrolysis of benzoylcholine, unlike that of acetylcholine, is inhibited by excess of substrate. Vincent, Lagreu & Parant (1953) have shown that the hydrolysis of benzoylcholine by human-serum cholinesterase is selectively inhibited by caffeine.

The estimations of serum cholinesterase in liver disease have shown that a very low enzyme activity is always found in chronic liver damage; although in patients with acute liver damage the assay frequently gave an indication of abnormality, it could not, in any sense, be used as a criterion of the function of the liver cells.

The change in specificity found in liver disease could in some cases be a more sensitive indication of liver dysfunction than the single assay against acetylcholine; but the sera from patients with acute liver damage in some instances behaved normally.

This change in specificity pattern observed in liver disease is probably due to a slight change in the configuration of the enzyme. It seems unlikely that there is present in human serum a family of enzymes having slightly different specificity patterns which are produced in different relative amounts in liver disease, since the specificity pattern shown by Augustinsson (1949) for a purified preparation of cholinesterase was similar to that of whole serum. Members of a family of enzymes might be expected to behave differently on fractionation; thus a purified preparation might show a different specificity pattern from that of whole serum.

As a result of these investigations it is suggested that a detailed comparison of the substrate specificity produced by diseased cells with that from normal cells offers a way of studying changes in the configuration of a protein which are more subtle than can be detected from other types of measurements.

SUMMARY

1. The rates of hydrolysis of acetyl- and benzoylcholine by normal sera, and by sera from patients having chronic or acute liver damage, have been compared. Activity/ml. of serum was always decreased in chronic liver damage but was frequently found to be within the normal range in acute liver damage.

2. A comparison of the relative rates of hydrolysis of acetyl-, propionyl- and butyryl-choline by sera from normal persons and by sera from patients with chronic liver damage and from patients with acute liver damage showed that, in the five cases of chronic liver damage studied, the preferred substrate was always propionylcholine. This was so also in one case of acute liver damage, but the preferred substrate for the sera from the other five cases of acute liver damage studied was butyrylcholine. In all the sera examined, normal and pathological, the relative rates of hydrolysis of acetylcholine and butyrylcholine were constant.

3. The activation energies and pH optima for the hydrolysis of acetylcholine and benzoylcholine were measured. The values obtained on the sera from patients having chronic liver damage do not differ greatly from those obtained with normal sera.

My thanks are due to Professor N. H. Martin for his advice during the course of this work and for obtaining and selecting the sera which were examined. I am grateful to the Governors of St George's Hospital for a Smith's Research Studentship, during the tenure of which this work was carried out.

REFERENCES

Adams, D. H. & Whittaker, V. P. (1949). *Biochim. biophys. Acta*, **3**, 358.
Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
Arrhenius, S. (1889). *Z. phys. Chem.* **4**, 226.
Augustinsson, K.-B. (1949). *Arch. Biochem.* **23**, 111.
Callaway, S., Davies, D. R. & Rutland, J. P. (1951). *Brit. med. J.* ii, 812.
Glick, D. (1937). *Biochem. J.* **31**, 521.
Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Counc., Lond.*, no. 286.
Kekwick, R. G. O. (1955). Ph.D. Thesis: University of London.
Kekwick, R. G. O., Mackay, M. E. & Martin, M. H. (1953). *Biochem. J.* **53**, xxxvi.
McArdle, B. (1940). *Quart. J. Med.* **33**, 107.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Shukuya, R. (1953). *J. Biochem., Tokyo*, **40**, 135.
Surgenor, D. M. & Ellis, D. (1954). *J. Amer. chem. Soc.* **76**, 6049.
Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S. & Gibson, D. M. (1949). *J. Amer. chem. Soc.* **71**, 1223.
Vincent, D., Lagreu, R. & Parant, M. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 449.
Wilson, A., Calvert, R. J. & Geoghegan, H. (1952). *J. clin. Invest.* **31**, 815.

The Biochemistry of the Nitrifying Organisms

6. THE EFFECT OF OXYGEN CONCENTRATION ON NITRITE OXIDATION IN THE PRESENCE OF DIFFERENT INORGANIC IONS*

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(*Received* 6 *November* 1959)

Lees & Simpson (1957) reported that the cyanate ion was a strong inhibitor of nitrite oxidation by *Nitrobacter*. We have now found that this inhibition by cyanate, and by certain other inorganic ions, may be eliminated or even replaced by a stimulation if the oxygen concentration in the *Nitrobacter* suspension medium is decreased. A preliminary account of some of this work has already appeared (Butt & Lees, 1958).

MATERIALS AND METHODS

Culture methods. *Nitrobacter* cells were grown and harvested by methods essentially similar to those described by Lees & Simpson (1957). The main differences between the original techniques and those used in the present work were: (*a*) the 10 l. culture bottles were replaced by a 100 l. stainless-steel tank fitted with a bubbler tube to supply sterilized air and a 6 in. stainless-steel paddle rotating at 1450 rev./min. to ensure adequate aeration and mixing of the culture; (*b*) the concentration of inorganic salts (apart from nitrite) in the medium was decreased to one-tenth of the original value since it has been found (L. Herzenberg, personal communication) that *Nitrobacter* grows more readily and is more active in oxidizing nitrite when it is cultured in the more dilute medium.

Measurement of nitrite oxidation. In each experiment 0·5 ml. of a washed suspension of *Nitrobacter* cells was placed in the side arm of a Warburg flask; after temperature equilibration for 15 min. at 30°, the cells were tipped into the main chamber containing the other reactants in 2·5 ml. of 0·03 M-phosphate buffer, pH 7·5, and the oxygen uptake was measured over a suitable period.

The required oxygen concentrations were obtained by flushing with mixtures of oxygen and nitrogen prepared by displacement of water from a graduated reservoir.

Reagents. Sodium nitrite used for culturing *Nitrobacter* was of technical grade; sodium arsenite was A.R. grade; all other reagents were as those used by Lees & Simpson (1957).

RESULTS

Nitrate, at a concentration of 33 mM, inhibited nitrite oxidation at high oxygen concentrations but stimulated it at lower oxygen concentrations (Table 1); this result is typical of several examples obtained with different nitrite and nitrate concentrations. Similar results were obtained with

* Part 5: Lees & Simpson (1957).

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Table 1. *Effect of nitrate, arsenite and cyanate on nitrite oxidation by* Nitrobacter *at different oxygen concentrations*

In each Warburg vessel there was 2·5 ml. of 0·03 M-phosphate buffer, pH 7·5, containing nitrite and nitrate, arsenite or cyanate. The gas phase in each vessel was changed by flushing with the appropriate mixture of oxygen and nitrogen and, after temperature equilibration at 30°, 0·5 ml. of *Nitrobacter* suspension (containing 0·25–0·5 mg. dry wt. of cells) was added from the side arm. In the experiment with arsenite, the initial nitrite concentration was 17 mM and in all other experiments 33 mM. Results are expressed as a percentage of the control value in the absence of inhibitor.

		Oxygen in gas phase (%)						
		1·25	2·5	5·0	7·5	10	15	20
Compound	Concentration (mM)	Percentage of control value						
Nitrate	33	200	233	109	68	66	—	—
Arsenite	8	—	167	191	—	107	82	73
Cyanate	0·3	214	104	74	60	36	—	—
	0·2		130					50
	0·3		145					34
	0·5		118					24
	0·7		97					18
	1·0		54					8

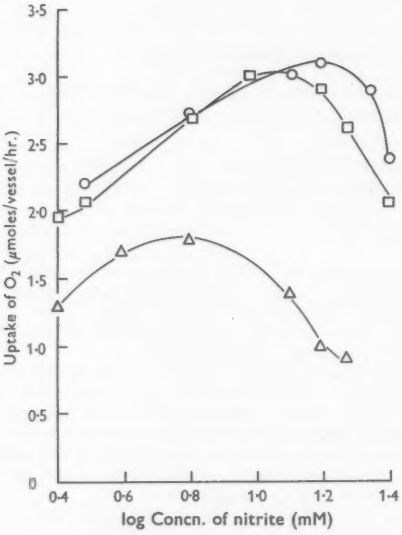


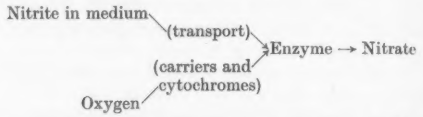
Fig. 1. Relationship between nitrite concentration and the rate of nitrite oxidation by *Nitrobacter* at different oxygen concentrations. In each vessel there was 2·5 ml. of 0·03 M-phosphate buffer, pH 7·5, containing the nitrite. In the side arm there was 0·5 ml. of washed *Nitrobacter* suspension containing 0·25–0·5 mg. dry wt. of cells. The gas phase in each vessel was changed by flushing with the appropriate mixture of oxygen and nitrogen before temperature equilibration at 30°. Oxygen concentrations used were: ○, 20%; □, 10%; △, 2·5%.

arsenite or cyanate in place of nitrate (Table 1); chloride and sulphate had little effect at any oxygen concentration tested. The effect on nitrite oxidation of varying cyanate concentrations at low and normal oxygen concentrations is also shown in Table 1.

As Meyerhof (1917) showed, the rate of nitrite oxidation by *Nitrobacter* first of all increases as the nitrite concentration is increased, reaches a maximum and then begins to diminish as the nitrite concentration is further increased; typical curves for the variation of the rate of nitrite oxidation with nitrite concentration at various oxygen concentrations are shown in Fig. 1. This 'humped' type of curve is characteristic of enzyme systems in which there is inhibition by excess of substrate. Fig. 1 also shows how the optimum nitrite concentration for nitrite oxidation varies with oxygen concentration.

DISCUSSION

The site of the inhibition by excess of nitrite may be either the enzyme (e.g. by formation of abnormal enzyme–substrate complexes) or a component of the electron-transport system involved in nitrite oxidation. The fall in optimum nitrite concentration as the oxygen concentration is lowered (Fig. 1) may be explained if it is assumed that a transport system carries nitrite into the cell. The type of mechanism suggested is:



At moderate nitrite concentrations (up to about 20 mM) and normal oxygen concentrations (20% oxygen in the gas phase) the rate at which nitrite is transported into the cell and the rate at which it is oxidized via the carrier–cytochrome system are assumed to be roughly equal, so that there is no appreciable accumulation of nitrite within the cell. When the oxygen concentration is lowered, however, nitrite should accumulate at the enzyme surface and inhibit nitrite oxidation. At lower oxygen concentrations therefore the optimum oxidation rate should occur at a lower nitrite concentration in the medium.

Nitrate, arsenite and cyanate (being somewhat similar in chemical structure to nitrite) may interfere with the nitrite-transport system. By lowering the rate of transport of nitrite, these ions decrease the rate of nitrite oxidation at normal oxygen concentrations. At lower oxygen concentrations, however, they prevent accumulation of nitrite within the cell and thus stimulate nitrite oxidation.

We have, of course, no direct proof that these explanations are correct. They can be proved only when it becomes possible to separate the ion-transport mechanisms of *Nitrobacter* (if they exist) from the nitrite-oxidizing system. Since Aleem & Alexander (1958) claim to have obtained from *Nitrobacter* a cell-free system capable of oxidizing nitrite, proof or disproof of the validity of our explanations may be obtained soon.

SUMMARY

1. A study has been made of the influence of nitrite, and of certain other ions, on nitrite oxidation by *Nitrobacter* at normal and lowered oxygen concentrations.

2. Ions similar in structure to nitrite (nitrate, arsenite and cyanate) inhibited nitrite oxidation at normal oxygen concentrations and stimulated nitrite oxidation at decreased oxygen concentrations. Other ions (chloride and sulphate) had little effect at any oxygen concentration.

3. It was confirmed that nitrite inhibited its own oxidation. The nitrite concentration at which

a maximum rate of oxidation occurred decreased as the oxygen concentration was decreased.

We wish to thank Professor W. O. Kermack, F.R.S., for his advice and encouragement throughout this work. One of us (W.D.B.) also wishes to acknowledge the receipt of grants from the Ministry of Education and from Aberdeen University.

REFERENCES

Aleem, I. H. & Alexander, M. (1958). *Bact. Proc.* p. 8.
Butt, W. D. & Lees, H. (1958). *Biochem. J.* **69**, 3P.
Lees, H. & Simpson, J. R. (1957). *Biochem. J.* **65**, 297.
Meyerhof, O. (1917). *Pflüg. Arch. ges. Physiol.* **166**, 240.

Biochem. J. (1960) **76**, 427

The Pyridine Nucleotide Metabolism of Mitochondria Incubated with and without Added Substrates and Metabolic Inhibitors

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The experiments described previously (Birt & Bartley, 1960*a*, *b*) have shown that the amounts and state of reduction of the endogenous pyridine nucleotides of rat-liver mitochondria are controlled by the components of the incubating medium. This paper is concerned with a general study of the effects of the temperature of incubation, the addition of various substrates, 2:4-dinitrophenol and amytal on the behaviour of the pyridine nucleotides in these particles; in particular, factors altering the dynamic steady-state levels of reduced nucleotide (by changing rates of oxidation or reduction of the coenzymes, or both) have been investigated.

MATERIALS AND METHODS

The chemicals used in the preparation of media for the isolation and incubation of rat-liver mitochondria and the details of the preparative procedure have been described previously (Birt & Bartley, 1960*a*). The mitochondria were isolated in a medium containing sucrose (0·25M) plus nicotinamide (25 mM) and incubated in a 'saline' medium containing KCl (0·1M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer (pH 7·4, 50 mM), $MgSO_4$ (5 mM), sodium ethylenediaminetetra-acetate (EDTA, 1 mM), adenosine triphosphate (ATP, 1 mM) and nicotinamide (25 mM).

The methods of incubation and estimation of O_2 uptake and pyridine nucleotide analysis were as described by Birt & Bartley (1960*b*) and Bassham, Birt, Hems & Loening (1959).

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RESULTS

Changes in pyridine nucleotides during incubation without added substrates at different temperatures

A number of mitochondrial suspensions without added substrate were incubated at temperatures in the range 0–30° (Table 1). During 1 hr. at both 0° and 10°, there was no destruction of coenzyme and no decline in reduced pyridine nucleotide; indeed, at both temperatures there was a tendency for the amount of reduced diphosphopyridine nucleotide (DPNH) to increase slowly, though the level of reduced triphosphopyridine nucleotide (TPNH) did not change significantly. At and above 15°, however, the total coenzyme level declined by approximately 20% in 1 hr.; di- and tri-phosphopyridine nucleotides (DPN^+ and TPN^+) disappeared at similar rates. There was also a net loss of reduced coenzyme during 1 hr. The DPNH level usually fell, in 15–30 min., to a value below which little further decline occurred, but the oxidation of TPNH commonly continued throughout the hour of incubation, especially at 20° (similar data were obtained with mitochondria incubated at 25° without substrate). The last two columns of Table 1 indicate the relative changes occurring in the proportions of the di- and tri-phosphopyridine nucleotides present in the reduced form; values greater than 1·0 were the result of a decrease below the initial proportion of reduced coenzyme; values less than 1·0 of an increase over the initial proportion of reduced coenzyme. At 15° and above, the proportional change after incubation for 5 min. was always greater in the triphosphopyridine

Table 1. *Effect of temperature on the oxidation of endogenous reduced nucleotides in different mitochondrial preparations*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Incubations were carried out in 'saline' medium, with gentle shaking. In this and subsequent tables DPN = sum of oxidized and reduced diphosphopyridine nucleotides ($DPN^+ + DPNH$); TPN = sum of oxidized and reduced triphosphopyridine nucleotides ($TPN^+ + TPNH$); $PN^+ = DPN^+ + TPN^+$; PNH = DPNH + TPNH; PN = PN^+ + PNH.

Temp.	Time (min.)	DPN	TPN	PN	$\frac{DPNH}{DPN^+}$	$\frac{TPNH}{TPN^+}$	$\frac{PNH}{PN^+}$	$\frac{DPNH}{DPN^+}$ at time 0 min. / $\frac{DPNH}{DPN^+}$ at time shown	$\frac{TPNH}{TPN^+}$ at time 0 min. / $\frac{TPNH}{TPN^+}$ at time shown
0°	0	1·90	0·86	2·76	0·49	7·5	1·1	1·0	1·0
	5	1·90	0·95	2·85	0·49	6·9	1·0	1·0	4·0
	15	2·20	0·91	3·11	0·59	10·4	1·1	0·83	2·7
	30	2·00	1·05	3·05	0·76	6·9	1·4	0·64	4·0
	60	2·10	0·76	2·86	0·70	3·8	1·0	0·70	7·2
10	0	1·55	1·05	2·60	0·64	4·0	1·3	1·0	1·0
	5	1·65	1·10	2·75	0·60	3·4	1·1	1·1	1·2
	15	1·90	0·98	2·88	0·99	3·3	1·4	0·65	1·2
	30	1·65	1·00	2·65	0·79	8·1	1·6	0·81	0·49
	60	1·85	0·88	2·73	1·03	3·4	1·5	0·62	1·2
15	0	2·15	2·00	4·15	0·97	6·6	2·1	1·0	1·0
	5	1·70	1·60	3·30	0·45	3·0	1·1	2·2	2·2
	15	1·60	1·56	3·15	0·21	0·6	0·37	4·6	11·0
	30	1·55	1·65	3·20	0·19	0·4	0·30	5·1	17·0
	60	1·50	1·50	3·00	0·24	0·5	0·35	4·0	13·0
20	0	2·00	1·95	3·95	0·71	6·4	1·8	1·0	1·0
	5	1·85	1·90	3·75	0·75	4·7	1·7	0·95	1·4
	15	1·65	1·50	3·15	0·27	1·4	0·65	2·6	4·6
	30	1·65	1·60	3·25	0·23	0·83	0·47	3·1	7·7
	60	1·70	1·75	3·45	0·25	0·50	0·37	2·8	13·0
30	0	2·45	2·30	4·75	1·10	4·1	1·9	1·0	1·0
	5	2·25	2·45	4·70	2·3	8·7	3·9	0·48	0·47
	15	2·35	2·10	4·45	0·54	1·2	0·8	2·0	3·4
	30	1·95	2·20	4·15	0·50	1·4	0·9	2·2	2·9
	60	1·75	1·35	3·10	0·04	0·12	0·1	28·0	34·0

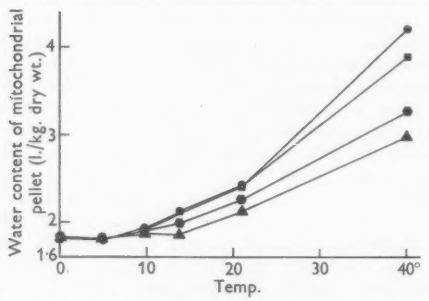

Fig. 1. Effect of temperature on the time course of mitochondrial swelling. Samples of mitochondrial suspension (2 ml. in 0·25M-sucrose) were maintained for the times (▲, 15 min.; ●, 30 min.; ■, 45 min.; ●, 60 min.) and temperatures indicated. The ratio of water to dry weight of the mitochondrial pellets obtained by high-speed centrifuging of the suspension was determined according to Werkheiser & Bartley (1957).

nucleotide, i.e. the ratio $TPNH/TPN^+$ declined more rapidly than the ratio $DPNH/DPN^+$. Further, in all the experiments quoted, the amounts of TPNH disappearing in each interval of time were greater than those of DPNH.

Even when there was a net oxidation of the reduced coenzyme after incubation for 1 hr. (i.e. at 15° and above), there was sometimes an initial transient increase in the amount of reduced nucleotides (Table 1, experiments at 20° and 30°), followed by their oxidation. This was most marked at 30°. The initial reduction of oxidized coenzymes in a mitochondrial suspension incubated without added substrate was enhanced greatly by increasing the concentration of mitochondria in the suspension. In all the experiments quoted in Table 1, the preparations contained 20–30 mg. of dry matter/ml.; the results obtained with a suspension having 45 mg. of dry matter/ml. showed that, on incubation, there was a large increase in the level of reduced coenzymes, and the restoration of the original ratio of reduced to oxidized nucleotide did not occur until after 30 min. The reduction of TPN^+ lagged behind that of DPN^+ but the ensuing oxidation of TPNH was more rapid than that of DPNH.

The series of incubations (Table 1) indicated that a rise from 0° to 10° did not affect the coenzymes

measurably, but that a rise of a further 5° produced all the changes characteristic of actively oxidizing mitochondria, though the final levels (after incubation for 60 min.) of reduced coenzymes were somewhat higher than at 20° and above. There were analogous temperature-dependent changes in the water content of rat-liver mitochondria suspended not in the 'saline' medium but in 0·25M-sucrose (Fig. 1). This is further evidence for a relationship between the maintenance of mitochondrial structure and the retention of reduced pyridine nucleotide (see Lehninger, Ray & Schneider, 1959).

All subsequent incubations were done at 25°, for although at this temperature the changes in oxidized and reduced coenzyme levels were sufficiently large to be measured accurately, the mitochondria did not undergo the almost complete loss of reduced nucleotides that occurred at 30°.

Oxygen uptake of mitochondria incubated in 'saline' medium without substrate

Table 2 presents the levels of oxidized and reduced coenzymes and the initial (calculated for the first 15 min.) and final (calculated for last 15 min.) rates of oxygen uptake. Expts. 1–4 show typical results obtained in the absence of substrate; the rate of oxygen uptake generally declined during the hour of incubation from an initial Q_{O_2} value of about 3. At the same time, the ratio of reduced to oxidized pyridine nucleotides diminished, but did not decrease below about 0·1. Exceptional experiments are Expts. 5–7, where there was an increase in the rate of oxygen consumption, and an almost complete disappearance of reduced coenzyme. In one experiment (Expt. 4) the rate of oxygen uptake declined although the final ratio of reduced to oxidized coenzyme was much less than 0·1. These data suggest that pyridine nucleotide-dependent respiration declines as the total amount of coenzyme and of endogenous substrate available to reduce it decreases, and is later replaced by other types of oxidative processes.

In some experiments the initial rate of oxygen consumption was as high as 6–9 μl./mg. dry wt./hr. There was no simple relationship between the initial rate of respiration and the ratio of reduced/oxidized coenzyme in the isolated mitochondria.

Effect of added substrates on the pyridine nucleotides and oxygen uptake of mitochondria

All the metabolites added (Table 3) increased the rate of oxygen consumption; considering only the stimulations produced by compounds dependent on DPN^+ or TPN^+ for their oxidation, the order of decreasing effectiveness was α-oxoglutarate, citrate, pyruvate plus malate, pyruvate, β-hydroxybutyrate and malate. The rates of oxygen consumption with the last-named four were constant during incubation for 1 hr.; that with citrate declined by 50%. α-Oxoglutarate, glutamate, pyruvate plus malate and β-hydroxybutyrate all increased the ratio of total reduced to total oxidized coenzyme over the initial value for isolated mitochondria before the first sample could be removed (at 'zero' time, usually 0·5–1·5 min. after addition of the substrate, at a temperature of about 5°), whereas citrate, malate and pyruvate did not. The greater the effect of the added substrate on the ratio of reduced to oxidized pyridine nucleotides, the greater was the effect on the rate of respiration in the first 15 min. Subsequently, all the substrates maintained the ratio for 1 hr. at higher values than that observed in the corresponding suspension without substrate; it was not possible to relate such decreases in the ratio to decreasing rates of respiration (see especially the experiments with citrate, malate and hydroxybutyrate, Table 3).

The effect of two substrates not dependent on pyridine nucleotides for their oxidation was also tested. One, choline, gave the maximum increase

Table 2. *Endogenous respiration and the ratios of reduced to oxidized mitochondrial nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake, as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°; all incubations were in 'saline' medium. See also protocol to Table 1.

	PN^+			PNH			PNH/PN^+			Uptake of O_2 at end of period	
Time (min.) ...	0	15	60	0	15	60	0	15	60	15	60
Expt. no.											
1	1·10	1·45	1·45	1·40	0·75	0·30	1·3	0·52	0·21	2·4	1·8
2	1·70	3·00	2·90	2·65	1·50	0·42	1·6	0·50	0·15	2·0	1·4
3	1·40	2·55	3·10	2·70	0·80	0·54	1·9	0·31	0·17	2·4	1·2
4	2·00	2·90	3·00	1·80	0·75	0·10	0·9	0·26	0·03	3·2	0·8
5*	2·00	3·10	3·20	1·70	0·30	0·16	0·9	0·10	0·05	0·1	3·0
6	1·35	2·45	2·40	1·85	0·74	0·14	1·4	0·30	0·06	3·0	6·0
7	1·85	3·15	3·30	2·60	0·76	0·10	1·4	0·24	0·03	2·4	2·8

* No ATP in 'saline' medium.

in respiration only after a lag phase; it had no effect on the ratio of reduced to oxidized coenzyme, which decreased as it did in the absence of added substrate. The second, succinate, produced a very pronounced increase in the rate of oxygen consumption during 15 min., though the increased level was not maintained throughout the incubation, and the decline in the respiration rate (68 % in 1 hr.) was greater than with any other substrate. This decline may have been due in part to the rapid removal of the substrate; if it is assumed that only succinate was oxidized, the data of Table 3, Expt. 3, show that about 53 % of the succinate was removed in 15 min. The production of even small amounts of oxaloacetate by the further metabolism of the fumarate produced may also have inhibited succinate oxidation. Succinate also produced the highest ratio of reduced to oxidized coenzyme observed in this series of experiments; the amount of reduced nucleotide had been greatly increased before the first sample could be taken. Higher levels of DPNH and TPNH were found during incubation for 1 hr. with succinate than with any other substrate tested.

An attempt was made to follow the time course of the reduction of the coenzymes after the addition of succinate. However, even in 0·5 min. considerable reduction had occurred, and the ratio had risen to its maximum value after incubation for only 6 min. Thus the rapidity of the reaction prevents the accurate measurement of the increase in reduced coenzyme by the analytical method used (compare the results obtained with the recording spectrophotometer by Chance, 1954). When pyruvate was used as substrate (Table 8, column 11) the net reduction of the nucleotides was sufficiently slow to be followed.

Effect of substrate on the pyridine nucleotides on the 'fluffy layer'. The pyridine nucleotides of the 'fluffy layer' responded to added pyruvate and succinate in the same way as did the mitochondria, succinate being more effective in reducing the coenzymes than pyruvate; there was no decline in the ratio reduced/oxidized nucleo-

Table 3. *Pyridine nucleotides of mitochondria incubated with added substrates*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°; concn. of all added substrates, 10 mM. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Expt. no.	Added substrate	Time (min.)	PN+	PNH	PN	PNH / PN+	Uptake of O_2 at end of time	Loss of nucleotides (%)
	Nil	0	1·40	2·15	3·55	1·55	—	—
		15	2·55	1·05	3·60	0·41	2·3	0
		60	2·60	0·37	2·96	0·14	1·5	17
1	Citrate	0	1·40	2·00	3·40	1·40	—	—
		15	0·72	2·80	3·52	3·90	16	0
		60	0·72	1·90	2·62	2·60	7·8	24
2	α-Oxoglutarate	0	1·00	2·80	3·80	2·80	—	—
		15	0·66	3·15	3·81	4·80	20	0
		60	1·25	1·25	2·50	1·00	—	34
2	Glutamate	0	1·20	2·65	3·85	2·20	—	—
		15	0·93	2·95	3·88	3·15	—	0
		60	1·45	1·10	2·55	0·76	—	33
3	Succinate	0	0·59	2·80	3·39	4·80	—	—
		15	0·44	2·65	3·09	6·00	14·2	9
		60	0·62	1·90	2·52	3·10	4·6	23
4	Malate	0	1·25	2·00	3·25	1·60	—	—
		15	1·20	2·15	3·35	1·80	6·6	0
		60	1·30	1·00	2·30	0·77	6·0	29
4	Pyruvate	0	1·30	1·90	3·20	1·50	—	—
		15	0·82	2·55	3·37	3·10	8·0	0
		60	0·75	2·10	2·85	2·80	7·8	12
5	Pyruvate plus malate	0	1·15	2·85	4·00	2·50	—	—
		15	0·81	2·80	3·61	3·50	12·5	10
		60	0·90	2·20	3·10	2·45	10·7	22
6	β-Hydroxybutyrate	0	1·50	3·25	4·75	2·15	—	—
		15	1·90	2·70	4·60	1·40	6·9	5
		60	2·85	1·05	3·90	0·37	4·8	19
6	Choline	0	1·90	2·55	4·45	1·35	—	—
		15	3·20	0·75	3·95	0·23	4·0	11
		60	2·65	0·42	3·07	0·16	6·9	31

tide during incubation for 15 min. without added substrate.

Reduction of di- and tri-phosphopyridine nucleotides by added substrates. Separate analyses of DPN^+, DPNH, and TPN^+ and TPNH during incubation with citrate, α-oxoglutarate, pyruvate and succinate showed that of the three substrates reacting directly with pyridine nucleotides only α-oxoglutarate had an immediate effect in reducing DPN^+ and TPN^+. The amount of TPNH formed was always small. Pyruvate and citrate had no immediate effect but reduced both DPN^+ and TPN^+ in 15 min. There was no clear evidence for a preferential reduction of TPN^+ by citrate. The immediate reduction of the coenzymes after succinate addition involved both DPN^+ and TPN^+, as did the subsequent maintenance of a high ratio of reduced to oxidized coenzymes. With all four substrates, the amounts of TPN^+ in the mitochondria after 15 min. were extremely small.

Reduction of pyridine nucleotides in mitochondria pre-incubated without substrate. When mitochondria were allowed to oxidize their endogenous coenzymes during pre-incubation for 30 min. at 25° (Table 4) before addition of β-hydroxybutyrate, succinate or pyruvate plus malate (10 mM), the levels of both DPNH and TPNH increased during a further 5 min. of incubation. With each substrate the greater increase was in DPNH. The ratios of reduced to oxidized coenzyme with succinate and pyruvate plus malate (4·3 and 3·8 respectively) were similar to those found after short incubation of freshly isolated mitochondria (see Table 3), but with β-hydroxybutyrate the ratio was lower (1·3 in this experiment compared with 2·15 in Table 3). Moreover, in an experiment in which pre-incubation was continued for 40 min., the addition of 100 mM-β-hydroxybutyrate merely maintained, but did not increase, the levels of both DPNH and TPNH during a further 5 min. After 15 min. most of the coenzyme was oxidized. Thus it seems that whereas the enzymes responsible for dehydrogenating succinate and pyruvate plus malate retained their activity during pre-incubation, β-hydroxybutyric dehydrogenase was to some extent inactivated. The results of experiments on the reactivity of released nucleotides (Birt & Bartley, 1960*a*) and the rapid decline in the amount of reduced coenzyme described in Table 3 support this inference.

Effect of 2:4-dinitrophenol on the pyridine nucleotides of mitochondria

It has been suggested that the mechanism by which phosphorylation exerts a control on the respiration of mitochondria involves reactions of the pyridine nucleotides (see Chance & Williams, 1956). Preliminary experiments showed that in the presence of 2:4-dinitrophenol, pyruvate was less effective in maintaining the level of reduced pyridine nucleotides than was succinate. This was further tested with three different concentrations of dinitrophenol in the presence of succinate or pyruvate. At all concentrations dinitrophenol diminished the formation of reduced pyridine nucleotide on addition of substrate, but this effect was much greater with pyruvate than with succinate, especially at 10 μM-dinitrophenol. With this concentration (Table 5) when succinate was present the pyridine nucleotides were 95% reduced after incubation for 15 min. and 72% after 45 min. With pyruvate the comparable figures were 88 and 7·5% respectively. Even with

Table 4. *Reduction by β-hydroxybutyrate, succinate and pyruvate plus malate of the pyridine nucleotides of mitochondria pre-incubated without added substrate*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Temp. of incubation, 25°; concn. of β-hydroxybutyrate, succinate, pyruvate and malate each 10 mM. The mitochondria were pre-incubated in 'saline' medium alone for 30 min. at 25°, and the suspension was sampled before the addition of the substrates. Final samples for analysis were taken after a further incubation for 5 min. at 25°. See also protocol to Table 1.

	No substrate (30 min.)	β-Hydroxybutyrate (35 min.)	Succinate (35 min.)	Pyruvate plus malate (35 min.)
DPN^+	1·52	1·28	0·48	0·70
DPNH	0·76	1·04	1·76	1·56
DPN	2·28	2·32	2·24	2·26
$\frac{DPNH}{DPN^+}$	0·50	0·81	3·6	2·2
TPN^+	0·53	0·42	0·26	0·10
TPNH	0·97	1·23	1·44	1·48
TPN	1·50	1·65	1·70	1·58
$\frac{TPNH}{TPN^+}$	1·8	2·9	5·5	14·8

mM-dinitrophenol the pyridine nucleotides remained about 30% reduced throughout the incubation in the presence of succinate, whereas with pyruvate only 11·5% of the nucleotides was in the reduced form after 15 min. and 5% after 45 min. The increases in the respiration rates after 15 min. expressed as a percentage increase over the rate for mitochondria incubated without substrate (Table 5) produced by 1 mM-, 0·1 mM- and 10 μM-dinitrophenol were, for pyruvate, 2, 70 and 125% respectively; for succinate, the corresponding values were 180, 260 and 250%. All these rates decreased after 45 min. Throughout the incubation the maximum stimulation of respiration with pyruvate was produced by 10 μM-dinitrophenol, and with succinate by 0·1 mM-dinitrophenol.

In this experiment with succinate there was a 38-fold difference in the ratios of reduced to oxidized pyridine nucleotides but only a 25% difference in the respiration rate (compare values with succinate + mM- and 10 μM-dinitrophenol); a sevenfold difference in this ratio occurred without difference in the respiration rate (compare values with 0·1 mM- and 10 μM-dinitrophenol).

Further experiments with both substrates and different concentrations of dinitrophenol confirmed the general observations made above though the magnitude of some of the effects differed (Tables 6

Table 5. *Effect of different concentrations of* 2:4*-dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°; concn. of pyruvate and succinate, 10 mM. The initial mitochondrial suspension (0°, 0 min.) contained PN^+, 1·67; PNH, 3·20; PN, 4·87; PNH/PN^+, 1·9. 'Percentage loss' is the percentage decrease on the total amount of coenzyme below the initial total: 4·87 μm-moles/mg. dry wt. Without added substrate, the rates of O_2 consumption at 15 min. and 45 min. were 9·8 μl./mg. dry wt./hr. and 4·8 μl./mg. dry wt./hr. respectively. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

		15 min.						45 min.					
Substrate	Concn. of dinitrophenol (mM)	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2
Pyruvate	1	3·70	0·49	4·19	0·13	14	10·0	3·80	0·20	4·00	0·05	18	5·1
	0·1	2·80	1·25	4·05	0·45	17	16·4	3·20	0·20	3·40	0·06	29	5·4
	0·01	0·90	3·50	4·40	3·9	10	22·0	3·75	0·30	4·05	0·08	17	9·9
Succinate	1	3·05	1·45	4·50	0·48	8	27·6	3·20	1·20	4·40	0·38	9	18·4
	0·1	1·25	3·15	4·40	2·5	10	35·2	1·65	3·00	4·65	1·8	5	26·2
	0·01	0·25	4·55	4·80	18·0	0	34·5	1·30	3·30	4·60	2·5	6	18·5

Table 6. *Effect of* 2:4*-dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake, as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°; concn. of succinate and pyruvate, 10 mM. 'Percentage loss' is the percentage decrease in the total amount of coenzyme below the highest initial total: 4·90 μm-moles/mg. dry wt. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

Succinate

	No addition						With dinitrophenol (0·1 mM)					
Time (min.)	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2
0	1·25	3·60	4·85	2·9	—	—	2·35	2·10	4·45	0·90	—	—
15	0·63	3·90	4·53	6·2	6	14·6	2·90	1·90	4·80	0·66	0	17·2
60	1·50	2·50	4·00	1·7	17	9·6	2·85	1·00	3·85	0·35	14	15·6

Pyruvate

	No addition						With dinitrophenol (0·1 mM)					
Time (min.)	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2	PN^+	PNH	PN	PNH/PN^+	Loss (%)	Uptake of O_2
0	1·70	2·95	4·65	1·7	—	—	2·35	2·55	4·90	1·1	—	—
15	1·25	3·55	4·80	2·8	0	8·0	3·50	0·52	4·02	0·15	18	7·6
60	1·40	2·55	3·95	1·8	14	9·0	2·90	0·23	3·13	0·08	36	9·0

Table 7. *Effect of* 2:4-*dinitrophenol and succinate or pyruvate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°; concn. of succinate and pyruvate, 10 mM. 'Percentage loss' is the percentage decrease in the total amount of coenzyme below the highest initial total: 4·15 μm-moles/mg. dry wt. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

	Succinate											
	No addition						With dinitrophenol (10 μM)					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	0·81	3·30	4·11	4·1	—	—	1·05	3·10	4·15	3·0	—	—
15	0·78	3·05	3·53	3·9	7	15·6	1·10	2·90	4·00	2·6	3	21·2
60	0·96	2·00	2·96	2·1	28	5·5	1·10	1·85	2·95	1·7	28	13·0
	Pyruvate											
	No addition						With dinitrophenol (10 μM)					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	1·45	2·35	3·80	1·6	—	—	1·65	2·10	3·75	1·3	—	—
15	0·89	2·80	3·69	3·2	2	9·6	0·69	2·90	3·60	4·2	4	14·4
60	1·05	2·15	3·20	2·1	19	5·1	1·25	1·95	3·20	1·5	15	12·0

Table 8. *Effect of* 2:4-*dinitrophenol, pyruvate, malate and pyruvate plus malate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temp. of incubation, 25°. The Warburg vessels contained about 50 mg. dry wt. of mitochondria in a total volume of 3 ml. See also protocol to Table 1.

		15 min.					45 min.				
Substrate added	Concn. of dinitrophenol (mM)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Uptake of O_2
Nil	Nil	—	—	—	—	6·0	2·2	2·1	4·3	1·0	2·8
Pyruvate (10 mM)	Nil	2·1	1·9	4·0	0·9	10·2	1·6	2·6	4·2	1·6	12·0
Pyruvate (10 mM)	0·1	3·7	0·15	3·85	0·04	19·3	3·7	0·18	3·88	0·05	9·0
Malate (10 mM)	Nil	1·2	3·5	4·7	2·9	6·6	1·3	3·1	4·4	2·4	2·4
Malate (10 mM)	0·1	1·2	3·6	4·8	3·0	22·6	1·7	2·3	4·0	1·4	22·2
Pyruvate (10 mM) plus malate (1 mM)	0·1	1·8	2·9	4·7	1·6	13·8	3·2	0·25	3·45	0·08	9·6
Pyruvate (10 mM) plus malate (10 mM)	0·1	0·6	4·2	4·8	7·0	22·6	1·6	2·4	4·0	1·5	20·4

and 7). Dinitrophenol (10 μM) decreased slightly the proportion of reduced coenzyme produced by succinate and pyruvate. With dinitrophenol (0·1 mM) succinate was less effective in reducing the pyridine nucleotides than in the experiment already described (Table 5), though it produced a greater amount of reduced coenzyme than did pyruvate under the same conditions (Tables 6 and 7). Both concentrations of dinitrophenol increased the respiration rate with succinate; but only the lower concentration had this effect with pyruvate, dinitrophenol (0·1 mM) actually decreasing the initial rate.

In summary, although the response the of pyridine nucleotides to different concentrations of dinitrophenol and succinate or pyruvate is somewhat variable, it was apparent that:

(i) The reduction of the coenzymes was more readily inhibited by dinitrophenol (1 mM–10 μM) in the presence of pyruvate than in the presence of succinate. Respiration with succinate was always stimulated by both 0·1 mM- and 10 μM-dinitrophenol, but with pyruvate, concentrations of 0·1 mM and greater may depress respiration.

(ii) It was possible to obtain high values (2·5–18) for the ratio reduced/oxidized coenzyme with succinate plus 0·1 mM- and 10 μM-dinitrophenol.

Effect of dinitrophenol with pyruvate plus malate. As pyruvate plus malate produce high ratios of reduced to oxidized pyridine nucleotides (see Table 3), the effect of dinitrophenol in combination with these two substrates was tested. Table 8 shows that:

(i) Dinitrophenol (0·1 mM) had a relatively small effect on the reduction of pyridine nucleotides by malate after either 15 or 45 min. (compare Table 6).

(ii) When malate (1 mM) was added with pyruvate (10 mM) and dinitrophenol, the level of reduced nucleotide was maintained only during 15 min. of incubation; whereas with 10 mM-malate and -pyruvate the amount of reduced nucleotide was almost identical, even after 45 min., with that produced by pyruvate without dinitrophenol. These results suggested that a decreased availability of C_4 acids was responsible, at least in part, for the effect of dinitrophenol on the reduction of the coenzymes by added pyruvate (see Discussion).

(iii) The rate of respiration with malate was less than with pyruvate (see also Table 3) but the stimulation of this rate by dinitrophenol (0·1 mM) was greater, especially after longer times (45 min.) of incubation. The stimulated rate was not increased when pyruvate was present also.

(iv) Thus in the absence of dinitrophenol, malate was much less effective than malate plus pyruvate in stimulating respiration and reducing pyridine nucleotides, but there was a close similarity, both in respiration rate and changes in pyridine nucleotides, between the behaviour of mitochondria incubated in malate with DNP and malate plus pyruvate with DNP.

Detailed analysis of pyridine nucleotides in the presence of dinitrophenol. The decrease in the observed levels of reduced pyridine nucleotides in the presence of dinitrophenol was due to a diminution in the amounts of both DPNH and TPNH. After incubation for 15 min. without added substrate, the ratio of reduced to oxidized nucleotide had declined from 0·9 to 0·04; TPNH disappeared more rapidly than DPNH. With pyruvate and dinitrophenol there was an increase after incubation for 15 min. in the amount of DPNH only; succinate increased both that of DPNH and TPNH slightly.

Effect of 5-ethyl-5-isoamylbarbiturate (amytal) on the pyridine nucleotides of mitochondria

The respiratory inhibitor amytal (Ernster, Jalling, Löw & Lindberg, 1955) enhanced the reduction of pyridine nucleotide occurring after incubation for 15 min. without substrate or with pyruvate, but not that with succinate (Table 9). Longer incubations showed that without added substrate the effect of amytal was to maintain the concentration of reduced pyridine nucleotides during the early part of an incubation, but to accelerate their oxidation later; sometimes the maintenance occurred only after an initial lag.

Table 9. *Effect of amytal without added substrate, and with pyruvate or succinate, on the mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Temp. of incubation, 25°; concn. of pyruvate and succinate, 10 mM; of amytal, 1 mM in all mitochondrial suspensions. See also protocol to Table 1.

	No substrate			Pyruvate		Succinate	
	Zero time (no amytal)	15 min. (no amytal)	15 min. (amytal)	15 min. (no amytal)	15 min. (amytal)	15 min. (no amytal)	15 min. (amytal)
PN^+	1·40	2·50	1·30	2·70	0·75	0·30	0·46
PNH	3·25	1·50	2·90	1·65	3·40	3·85	3·95
PN	4·65	4·00	4·20	4·35	4·15	4·15	4·41
$\frac{PNH}{PN^+}$	2·3	0·6	2·2	0·6	4·5	12·8	8·6
Loss (%)	—	14	9	6	11	11	5

Table 10. *Effect of amytal without added substrate on mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt.; temp. of incubation, 25°; concn. of amytal, 1 mM. See also protocol to Table 1.

	Zero time				15 min.				30 min.			
	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	PN^+	PNH	PN	$\frac{PNH}{PN^+}$
Without amytal	2·20	2·40	4·60	1·1	1·55	2·60	4·15	1·7	2·30	1·20	3·50	0·52
With amytal	—	—	—	—	1·50	2·40	3·90	1·6	1·60	2·40	4·00	1·50

	45 min.				60 min.			
	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	PN^+	PNH	PN	$\frac{PNH}{PN^+}$
Without amytal	2·30	0·83	3·13	0·36	2·35	0·90	3·25	0·38
With amytal	2·00	1·60	3·60	0·80	3·05	0·41	3·46	0·13

Thus in the experiment described in Table 10, amytal was without effect after 15 min. (compare Table 9), it doubled the proportion of reduced coenzyme (relative to the control suspension at comparable times) after both 30 and 45 min. and halved it after 60 min. Pre-incubation with amytal for 15 min. at 0° did not influence the response to the inhibitor during subsequent incubation at 25°. During the pre-incubation there was no loss of pyridine nucleotide but the proportion of the total coenzyme reduced increased from 63 to 71 % in the presence of the amytal.

With added pyruvate also, the maintenance of the level of reduced pyridine nucleotide by amytal was transient. The enhanced accumulation of reduced coenzyme was most pronounced after about 5 min. of incubation. Thereafter the difference in the pyridine nucleotides of the suspensions with and without amytal diminished, until after 1 hr. the levels of reduced nucleotide were the same in both.

DISCUSSION

The experiments described illustrate how the amounts of the four pyridine nucleotides within the mitochondria vary with the experimental conditions. The proportions of the pyridine nucleotides can be affected by changes in the structure of the mitochondria or by competition between substrates for the terminal pathway of oxidation. Thus the abrupt increase in the rate of oxidation of the pyridine nucleotides with increase of temperature is probably due to a structural change since the rate of swelling also shows this abrupt change.

The effect of substrate competition is illustrated by the experiments with added succinate. It is likely that reducing equivalents donated by succinate and by DPNH will compete for common rate-limiting components of the electron-transport chain (compare Wu & Tsou, 1955); judged by the rate of oxygen uptake succinate is an extremely effective hydrogen donor and will therefore compete successfully with other substrates. High levels of reduced coenzyme will therefore be rapidly established and maintained. The effectiveness of succinate as an inhibitor of DPNH oxidation is illustrated by the rapid increase in the amounts of reduced pyridine nucleotides (usually maximal by the time of the first analysis) which occurred when this substrate was added. Chance & Hollunger (1957) explain similar observations as the reversal of oxidative phosphorylation resulting from increases in the amounts of high-energy phosphates formed during the oxidation of succinate. They report that uncoupling agents prevent this reduction, whereas in some of the experiments described in this paper (e.g. Tables 5 and 6), even after 15 min. in 0·1 mM-dinitrophenol, a concentration which not only prevents the synthesis of ATP during succinate oxidation by mitochondria suspended in 'saline' medium but also decreases the pre-existing level (Klemperer, 1957), succinate can maintain a high level of reduced pyridine nucleotide. It seems unlikely that, under these conditions of incubation, the reduction of coenzyme results from a hydrogen transfer from succinate mediated by a high level of ATP. A decrease in the rate of oxidation of reduced pyridine nucleotides by respiratory-chain competition is a more likely cause of the observed increase in the proportion of reduced coenzyme. The relatively small total amount of reducing equivalents required to produce the extra reduced coenzyme formed was probably derived from a hydrogen donor with an oxidation–reduction potential favouring reduction of pyridine nucleotide (e.g. endogenous substrate or malate produced from succinate), rather than from succinate itself.

Effect of dinitrophenol and amytal on the pyridine nucleotides of mitochondria

During the course of incubation for 1 hr., dinitrophenol always shifted the dynamic steady state of the pyridine nucleotides towards oxidation. The results in Tables 5–8 revealed a distinct difference between the ability of pyruvate on the one hand and succinate or malate on the other to maintain reduced pyridine nucleotides in the presence of dinitrophenol. It is possible that the effects of dinitrophenol on pyruvate oxidation were not due solely to the uncoupling of respiration and phosphorylation, but also to a decrease in the rate at which pyruvate itself was oxidized. In the experiments where dinitrophenol (at concentrations of 1 and 0·1 mM) had the greatest influence on the reduced coenzymes, the rate of oxygen consumption with pyruvate was also decreased by the uncoupling agent (Table 6). These effects could be reversed by the addition of malate, either at 10 mM or, for a short time only, at 1 mM concentration, because the availability of oxaloacetate limited pyruvate oxidation and therefore coenzyme reduction. The inhibition of respiration which sometimes followed the addition of dinitrophenol (0·1 mM or greater) may have been due to an impaired ability of the mitochondria to synthesize C_4 intermediates from pyruvate (Utter & Kurahashi, 1954; Bartley & Avi-Dor, 1955).

No evidence was obtained for the consistent release of substantial amounts of pyridine nucleotide from an 'inhibited' form during incubation with dinitrophenol (contrast Purvis, 1958; compare Baessler & Pressman, 1959).

The results obtained with amytal confirm the observations made by Ernster *et al.* (1955) and Chance & Hollunger (1957). However, it is

apparent that the time taken for amytal to exert its maximum effect in enhancing the reduction of pyridine nucleotides is variable, and that the effect itself is transient, being followed by changes in the mitochondrial behaviour considered as 'degenerative'.

Respiration and the pyridine nucleotide metabolism of mitochondria incubated without substrate

The rate of endogenous respiration of the mitochondria in the medium is relatively low, possibly due in part to the presence of EDTA and nicotinamide (compare Ziegler & Linnane, 1958). In four experiments in which the values for DPNH (as μm-moles/mg. dry wt.) were 0·86, 0·72, 0·48 and 0·44 with total reduced pyridine nucleotides 2·8, 2·4, 1·6 and 1·9 the initial respiratory rates (μl. of oxygen/mg. dry wt./hr.) were 4·3, 3·6, 1·3 and 1·5 respectively. Thus the amounts of both DPNH and total reduced pyridine nucleotide show a parallelism with the initial rates of oxygen uptake.

There was usually sufficient endogenous substrate to maintain or increase the level of reduced pyridine nucleotides for a limited time (up to 1 hr. at 15–25°). When there was an increase in respiration on prolonged incubation (compare Expts. 4 and 5, 6 and 7, Table 2; Expt. 6, Table 3) the pyridine nucleotides were almost completely oxidized. This relative independence of the amounts of reduced coenzyme and respiration rate suggests that the substrates being oxidized are not capable of reducing the pyridine nucleotides. It is possible that these changes are reflexions of mitochondrial 'degeneration' (Birt & Bartley, 1960*a*; Sacktor, 1954; compare also the increased ease of penetration of similar mitochondria by DPNH, Birt & Bartley, 1960*b*). The 'degenerative' changes were prevented by substrate and accelerated by amytal.

Effect of added substrate on the rate of respiration and reduction of pyridine nucleotides

The stimulation of the initial oxygen uptake that occurred on addition of substrate was directly related to the total measured level of reduced pyridine nucleotide (Table 3), and the relative magnitudes of the increases in the respiration rate with different substrates (with the exception of β-hydroxybutyrate) were the same as those reported by Ziegler & Linnane (1958) and D. Bellamy (unpublished work) for mitochondria in sucrose. The differences in the rates of net reduction of the coenzymes by different substrates could be due to an interaction of the pyridine nucleotides and enzymes released into the supernatant fluid, differences in the permeability of the mitochondria to different substrates, or differences in the activity of various dehydrogenases. Although certain enzymes are readily liberated from mitochondria in a form in which they can react with coenzymes and added substrates (Birt & Bartley, 1960*a*), the rapid and considerable reduction of pyridine nucleotide with succinate and β-hydroxybutyrate (both dehydrogenated by enzymes that are firmly bound to the mitochondria) and the slow reduction by malate (whose dehydrogenase can be made soluble) make it unlikely that much of this initial reduction occurs in the supernatant fluid. Ziegler & Linnane (1958), using the oxygen uptake of the mitochondria as a criterion for the ingress of a substrate, claim that undamaged particles in 0·5M-sucrose are impermeable to citrate and malate, but permeable to succinate, α-oxoglutarate, β-hydroxybutyrate, glutamate and pyruvate plus malate. The data of Table 3 can be reconciled with these observations, and it is possible that if the differences in the permeability of the mitochondria are indeed responsible for the observed differences in oxygen uptake the differences in the speed with which the coenzymes respond to the added substrate may also be attributed to this cause. However, Amoore (1958) has shown that mitochondria in 0·25M-sucrose at 0° have equal permeability to pyruvate, succinate and citrate. It would seem that a direct comparison of the penetration of different substrates into mitochondria at 25° is necessary for the resolution of this point. The slow reduction by pyruvate is greatly accelerated by the addition of malate, which is itself slowly dehydrogenated and is presumably without effect on the permeability to pyruvate. It is possible therefore that at least some of the variation in the rates of reduction is due to the different activities of the individual dehydrogenases under the conditions of incubation.

The oxidation of reduced coenzyme is unaffected by choline, which is oxidized by a flavoprotein (Rendina & Singer, 1958). Phosphorylation may occur during this oxidation (Rothschild, Cori & Barron, 1954), which therefore appears to be linked in some way to the electron-transport chain, though not in the same way as is succinate.

It has been shown that when there was a net reduction of pyridine nucleotides, the level of DPNH increased more rapidly than that of TPNH (see Table 1). In these experiments it was difficult to observe well-defined increases in the amount of TPNH, because very little of the triphosphopyridine nucleotide was present initially as TPN^+. However, experiments in which the level of TPNH was decreased by pre-incubation at 25° before the addition of substrate showed that succinate and pyruvate plus malate rapidly reduced both DPN and TPN; β-hydroxybutyrate was much less efficient in reducing the coenzymes, but it did increase the level of DPNH and, very slightly, that of TPNH. In all of these experiments the amount of DPNH produced was greater than that of TPNH.

A study of the time course of coenzyme reduction showed that when there was a net reduction of pyridine nucleotides the level of DPNH increased more rapidly than TPNH. Conversely, when the level of reduced coenzyme decreased, TPNH was oxidized faster than DPNH. As the four forms of the pyridine nucleotides are in a state of dynamic equilibrium involving reversible transhydrogenation (Stein, Kaplan & Ciotti, 1959), the following reactions may occur:

$$\left.\begin{array}{l} TPN^+ \xrightarrow{isocitrate} TPNH \\ DPN^+ \xrightarrow{substrate} DPNH \end{array}\right\} \text{Tricarboxylic acid cycle} \quad (1)$$

$$DPNH \xrightarrow[\text{transhydrogenase}]{TPN^+} TPNH \quad (2)$$

$$TPNH \xrightarrow[\text{transhydrogenase}]{DPN^+} DPNH + TPN^+ \quad (3)$$

Our experiments indicate that reactions (1) and (2) proceed when dehydrogenation is rapid relative to the rate of electron transport from DPNH to oxygen; reaction (3) occurs when dehydrogenation is relatively slow. Thus variations in the level of DPNH are minimized (see Table 1, and Birt & Bartley, 1960*a*), whether there is an excess or deficiency of substrate and TPNH acts as a reserve of reducing power, which is supplemented when the oxidation of substrates exceeds the capacity of the electron-transport chain and is depleted when there is little substrate. Since TPNH and TPN^+ readily pass through the mitochondrial membrane the function of the TPN^+-linked *iso*citric dehydrogenase may be to maintain TPNH despite a constant leakage; conversely TPNH made extramitochondrially may under some circumstances supplement the mitochondrial reserve.

Despite this interchange of the four forms of the pyridine nucleotides it seems that they do not have free access to the transhydrogenase within the particles since the quotient $[(TPNH \times DPN^+)/(TPN^+ \times DPNH)]$ is much greater than 1 (e.g. 6·7 from the results in Table 1; compare 0·7 in the presence of transhydrogenase isolated from ox heart by Kaplan, Colowick & Neufeld, 1953). The binding of different 'pools' of coenzyme by the dehydrogenases and the respiratory chain, and a pool of 'free' nucleotide (possibly containing more of the triphosphopyridine nucleotide) would be sufficient to account for this compartmentation.

As the activity of the transhydrogenase of rat-liver mitochondria is sufficient to allow rapid and reversible transfers of hydrogen between DPNH and TPNH (Stein *et al.* 1959), it is apparent that no conclusions about the oxidation and reduction of particular intramitochondrial coenzymes can be drawn without assays for all four pyridine nucleotides.

SUMMARY

1. A study has been made of the effect of temperature, added substrate and metabolic inhibitors on the amounts of oxidized and reduced di- and tri-phosphopyridine nucleotides in rat-liver mitochondria suspended in a 'saline' medium. The particles of the 'fluffy layer' behaved essentially in the same way as the mitochondria.

2. At and below 10°, the levels of reduced pyridine nucleotides increased slowly; reduced diphosphopyridine nucleotide accumulated more rapidly than reduced triphosphopyridine nucleotide during incubation with added substrate. Net oxidation (sometimes after an initial reduction) of coenzyme was detected only when the temperature was 15° and above, the rate of disappearance of reduced triphosphopyridine nucleotide being greater than that of reduced diphosphopyridine nucleotide. In more concentrated (twofold) suspensions of mitochondria the initial reduction was more pronounced.

3. Without substrate, the Q_{O_2} of the preparations (measured after 15 min. at 25°) was usually about 3 and the respiration rate declined during further incubation for 1 hr.

4. Added α-oxoglutarate, citrate, pyruvate plus malate, pyruvate, β-hydroxybutyrate and malate increased both the levels of reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide (maximum increase in ratio of reduced/oxidized coenzymes from 1·55 to 4·80, with added α-oxoglutarate; minimum increase from 1·55 to 1·80 with added malate) and the rates of oxygen uptake (maximum increase 8·5-fold, with α-oxoglutarate; minimum increase threefold, with malate), above the values found with freshly isolated mitochondria. All the substrates except citrate, pyruvate and malate reduced the nucleotides appreciably in less than 2 min.

5. Succinate produced the highest amounts of reduced coenzyme observed in suspensions of mitochondria, altering the levels in less than 30 sec. The increase in the ratio of reduced/oxidized coenzyme was about fourfold.

6. If the reduced nucleotides of isolated mitochondria were partially oxidized by pre-incubation for about 30 min. at 25° without added substrate, re-reduction of diphosphopyridine nucleotide (and, more slowly, triphosphopyridine nucleotide) could be demonstrated after the addition of pyruvate plus malate, β-hydroxybutyrate and succinate.

7. The reduction of mitochondrial pyridine nucleotides in the presence of pyruvate or without added substrate was less when dinitrophenol was added at concentrations of 10 μM or greater. Dinitrophenol (0·1 mM) was less effective in blocking reduction if malate was added with the pyruvate.

8. The reduction of coenzymes by malate and succinate was much less sensitive to dinitrophenol than that by pyruvate; relatively high ratios (2·5–18) of reduced to oxidized coenzyme could be obtained with concentrations of dinitrophenol of 10 μM and 0·1 mM.

9. The maintenance of reduced coenzyme by endogenous substrate and pyruvate was enhanced for a short time by amytal, the effect usually being maximal after about 30 min. of incubation. Subsequently there was a rapid decline in the amount of reduced coenzyme.

10. On the basis of the results obtained, it is proposed that the function of mitochondrial reduced triphosphopyridine nucleotide is that of a reserve of reducing power, which is supplemented when the oxidation of substrate exceeds the capacity of the electron-transport chain and which is utilized in the preservation of the level of reduced diphosphopyridine nucleotide when there is little oxidizable substrate. The significance of the data obtained with dinitrophenol for the formulation of the mechanism of the reduction of mitochondrial coenzymes by succinate and the evidence obtained for 'compartmentation' of these coenzymes is also discussed.

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REFERENCES

Amoore, J. E. (1958). *Biochem. J.* **70**, 718.
Baessler, K. H. & Pressman, B. C. (1959). *Fed. Proc.* **18**, 184.
Bartley, W. & Avi-Dor, Y. (1955). *Biochem. J.* **59**, 194.
Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. L. (1959). *Biochem. J.* **73**, 491.
Birt, L. M. & Bartley, W. (1960*a*). *Biochem. J.* **75**, 303.
Birt, L. M. & Bartley, W. (1960*b*). *Biochem. J.* **76**, 328.
Chance, B. (1954). *Science*, **120**, 767.
Chance, B. & Hollunger, G. (1957). *Fed. Proc.* **16**, 163.
Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
Ernster, L., Jalling, O., Löw, H. & Lindberg, O. (1955). *Exp. Cell Res.* Suppl. **3**, 124.
Kaplan, N. O., Colowick, S. P. & Neufeld, E. F. (1953). *J. biol. Chem.* **205**, 1.
Klemperer, H. G. (1957). *Biochim. biophys. Acta*, **23**, 404.
Lehninger, A. L., Ray, B. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 97.
Purvis, J. L. (1958). *Nature, Lond.*, **182**, 711.
Rendina, G. & Singer, T. P. (1958). *Biochim. biophys. Acta*, **30**, 441.
Rothschild, H. A., Cori, O. & Barron, E. S. G. (1954). *J. biol. Chem.* **208**, 41.
Sacktor, B. (1954). *J. gen. Physiol.* **37**, 343.
Stein, A. M., Kaplan, N. O. & Ciotti, M. M. (1959). *J. biol. Chem.* **234**, 979.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 821.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
Wu, C. Y. & Tsou, C. L. (1955). *Sci. Sinica*, **4**, 137.
Ziegler, D. M. & Linnane, A. W. (1958). *Biochim. biophys. Acta*, **30**, 53.

Biochem. J. (1960) **76**, 438

Exchange of Potassium Ions across a Concentration Difference by Isolated Rat-Liver Mitochondria

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Several experiments have been described which suggest that isolated mitochondria are capable of active transport (Spector, 1953; Macfarlane & Spencer, 1953; Stanbury & Mudge, 1953; Bartley & Davies, 1954; Mudge, 1955; Price, Fonnesu & Davies, 1956; Gamble, 1957; Bartley & Amoore, 1958). Nevertheless, it is difficult to prove that a given tissue can perform active transport. Rosenberg (1954) emphasized the necessity of showing that transport of solute from lower to higher electrochemical potential had occurred. The electrochemical potential should include terms representing both the chemical-concentration difference, and the electrical-potential difference, existing across the membrane (Ussing, 1949). Nothing seems to be known about the potential across the mitochondrial membrane, so the present argument is limited to consideration of the chemical-concentration differences. Even within these restricted terms of reference, however, the above-mentioned experiments are not entirely convincing, because of the following considerations.

Spector (1953), Stanbury & Mudge (1953), Mudge (1955) and Gamble (1957) measured move-

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ments of K^+ ions with respect to the nitrogen or dry-matter content of the mitochondria, without measuring the water content of the particles. They did not show that a concentration difference of K^+ ions existed between particles and medium during the incubation. The isolated mitochondria used by Macfarlane & Spencer (1953) and by Price *et al.* (1956) may have been considerably swollen, in view of later work by Werkheiser & Bartley (1957), and it is possible that the apparent movements of ions observed were largely due to changes in the fluid content of the mitochondria. In the experiments of Bartley & Davies (1954) the exchanges of Na^+ and K^+ ions between particles and medium proceeded so rapidly that it is doubtful whether an effective barrier to free diffusion of these ions was present; furthermore, their results on uptake of citric acid-cycle intermediates and of phosphate may have been influenced by adsorption, or by hydrolysis of labile phosphates during centrifuging (Amoore, 1958). Although some of the uptake of Mn^{2+} ions by mitochondria seemed to be an active process (Bartley & Amoore, 1958), these ions are strongly bound by the mitochondrial surface, so the concentration of free Mn^{2+} ions within the particles may have been no greater than that in the medium.

This paper describes experiments in which an attempt has been made to meet such criticisms.

METHODS

The general experimental procedure and analytical methods were as previously described (Werkheiser & Bartley, 1957; Amoore & Bartley, 1958). Rat-liver mitochondria were prepared by homogenizing and differentially centrifuging in 0·25 M-sucrose at 0°. At each stage of the experiment, the mitochondria in samples (2 ml.) of the suspension were centrifuged through 2·5 ml. of silicone fluid of sp.gr. 1·050. (No lower layer of aqueous 'fixative' was used.) When the centrifuge stopped, the tubes were placed in ethanol–solid CO_2 to freeze the contents. By this procedure, the mitochondrial pellets were promptly separated from the suspending medium, and no further redistribution of ions could occur. The mitochondrial pellets and supernatant fluid were recovered, extracted with 10% (w/v) trichloroacetic acid and the extract was analysed (Werkheiser & Bartley, 1957).

Extraction of lipid materials. Instead of being extracted with 10% trichloroacetic acid, two of the mitochondrial pellets were subjected to the following procedure for extracting lipids. This procedure was based on that of Solomon, Lionetti & Curran (1956), with modifications by Mr R. Hems of this Laboratory. The pellets, without being allowed to thaw, were 'freeze-dried' over P_2O_5 and extracted three times with $CHCl_3$–methanol (2:1, v/v) at 60°. The combined extracts were evaporated to dryness and the residue was extracted three times with $CHCl_3$ (previously dried over $CaCl_2$). The $CHCl_3$ extracts were combined and diluted to 10 ml. with $CHCl_3$ for determinations of radioactivity in a liquid counter. Then they were evaporated to dryness again, the $CHCl_3$-soluble residues were ashed with conc. HNO_3 and diluted for potassium estimations by flame photometry. The $CHCl_3$-insoluble residues were also ashed with conc. HNO_3 and assayed for radioactivity and for potassium.

Radioactivity determinations. The suspensions of mitochondria contained both [^{14}C]carboxypolyglucose (to measure extraparticulate fluid) and ^{42}KCl (to measure exchange of K^+ ions). The ^{42}K was measured with a liquid counter. The ^{14}C was measured with an end-window counter, after sufficient time had elapsed for the counting rate due to ^{42}K to have decreased to less than 1% of that due to ^{14}C. The water contents of both the mitochondria alone and of the whole mitochondrial pellet were calculated. The estimations of mitochondrial water were less consistent than were the estimations of total pellet water. This was probably because the estimations of extraparticulate fluid by means of [^{14}C]carboxypolyglucose were made without the refinements later found to be desirable (Amoore, 1958). Accordingly the concentrations of solutes in the water of the whole pellet were calculated and are shown in Table 1.

Measurements of pH. These were made with a Beckman pH meter model G, with the probe-assembly type 14400. The glass electrode was standardized with a phthalate buffer. The probe was inserted into the centrifuge tubes to measure the pH of the mitochondrial suspension before centrifuging. The buffering capacity of suspensions of mitochondria was estimated by titrating with N-HCl and measuring the pH with the glass electrode. The acid was delivered at a rate of 1 μl./min. from an Agla micrometer syringe (Burroughs Wellcome and Co.), which was driven by a synchronous motor (Smith's Sectric light-duty constant-speed motor). This arrangement is due to Mr A. Renshaw of this Laboratory (unpublished design). The mitochondrial suspension (2 ml.) was contained in a glass centrifuge tube placed in an ice bath, and was stirred by a gentle stream of CO_2-free air. The pH of the suspension was read at noted time intervals after starting the motor.

Definitions. In this work, the distributions of solutes were considered in terms of three different water spaces. Generally, the 'total water content of the pellet' was employed, as in Table 1. The 'internal water content of the mitochondria' represents the water volume which was not penetrated by [^{14}C]carboxypolyglucose. The 'inaccessible space of the mitochondria' represents the water volume which was not penetrated by sucrose. These quantities were calculated as described by Werkheiser & Bartley (1957) and by Amoore & Bartley (1958). Reasons have been given (Amoore & Bartley, 1958) for considering that only the 'inaccessible space' is surrounded by a barrier to free diffusion of sucrose and Na^+, K^+ and Cl^- ions.

EXPERIMENTAL AND RESULTS

In an experiment described previously (Amoore & Bartley, 1958) it was shown that when rat-liver mitochondria were re-suspended in ice-cold 0·125 M-potassium chloride containing ^{42}K, the endogenous potassium of the mitochondria exchanged only very slowly with the labelled K^+ ions of the medium. In 15 min. practically no exchange occurred; in 1 hr. less than one-tenth of the endogenous potassium exchanged, and even after 8 hr.

only three-quarters had exchanged. Mudge (1955) has shown that the rate of exchange of the endogenous potassium of rabbit-liver mitochondria with ^{42}K in the medium was about three times as fast at 25° as at 1°. In the following experiment a suspension of rat-liver mitochondria was incubated at 21°.

A medium composed of 0·04 M-potassium chloride and 0·25 M-sucrose was employed. A preliminary experiment showed that in this medium a concentration difference between mitochondria and medium of about 60 m-molal potassium was obtained, and that during 15 min. at 0° no exchange of mitochondrial potassium occurred.

Distribution of solutes at 0°. Rat-liver mitochondria were isolated in 0·25 M-sucrose and [^{14}C]-carboxypolyglucose was added. A sample of the suspension was centrifuged for analysis of the supernatant fluid and the mitochondrial pellet. The pellets had a water content of 2·74 l./kg. of *M*, where *M* represents mitochondrial dry weight (Table 1). The pellets contained 38·2 m-molal potassium and 5·6 m-molal chloride. Potassium chloride (1·5 M) was added to the suspension so as to produce a final concentration of about 0·04 M-potassium chloride. From here until the end of the experiment the mean concentration of sucrose in the supernatant medium was 245 m-molal, and the mean concentration of potassium chloride was 42·0 m-molal. After allowing 20 min. for equilibration, the mitochondrial pellets obtained had a decreased water content of 2·48 l./kg. of *M*, and increased concentrations of potassium and chloride (74·7 and 34·7 m-molal respectively). The excess of concentration of potassium, which is the difference between the concentrations of potassium in pellet and medium, was only a little less than it was before potassium chloride was added to the medium. The concentrations of both sucrose and chloride were substantially less than those present in the medium.

At time 24 min., a trace of ^{42}KCl was added to the suspension, and samples were immediately centrifuged. From the specific activity of the potassium in the supernatant fluid and in the mitochondrial pellet it was concluded that the labelled potassium reached a concentration of only 36·1 m-molal in the pellet. The difference between this and the total concentration of potassium in the pellet

Table 1. *Effect of incubation at* 21° *on the exchange of mitochondrial potassium with* ^{42}K *in the medium*

A suspension of rat-liver mitochondria in 0·25 M-sucrose (34 ml., containing 0·5 % of [^{14}C]carboxypolyglucose) was kept at 0° in a stoppered 50 ml. measuring cylinder. At time 0 min., two samples (2 ml.) were withdrawn and centrifuged at 0° for analysis of the mitochondrial pellet and supernatant fluid. At time 1 min., 0·80 ml. of 1·5 M-KCl was added to the remaining 30 ml. of suspension, and at time 20 min. two more samples were centrifuged at 0°. At time 24 min., 2·6 ml. of 0·04 M-^{42}KCl in 0·25 M-sucrose was added to the remaining 26 ml. of suspension, and 1 min. later three samples were centrifuged at 0°. (The pellet from one of these samples was preserved frozen for extraction of lipids.) At time 29 min., the remaining 22 ml. of suspension was poured into a 150 ml. conical flask (open to the air) placed in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill., U.S.A.) containing water at 21°. Two samples were immediately withdrawn and centrifuged at room temperature. Single samples were subsequently withdrawn and centrifuged (at room temperature) at times 35, 40, 45, 50 and 70 min. Finally, at time 90 min., two samples were centrifuged and the pellet from one of these samples was preserved frozen for extraction of lipids. The table shows means when duplicate pellets were analysed. Throughout the experiment, the mean concentration of sucrose in the supernatant medium was 245 m-molal, and (apart from the first sample) the mean concn. of KCl was 42·0 m-molal. The 'excess' of potassium in the pellet represents the difference between the total concentration of potassium in the pellet and that in the medium. The 'unexchanged' potassium in the pellet represents the difference between the total concentration of potassium and the concentration of ^{42}K—labelled potassium in the pellet. *M* represents mitochondrial dry wt.

Time (min.)	Temp.	Internal water content of mitochondria (l./kg. of *M*)	Total water content of pellet (l./kg. of *M*)	Concn. of solutes in pellet (m-molal)					
						Potassium			
				Sucrose	Chloride	Total	Excess	^{42}K	Unexchanged
0	0°	1·71	2·74	178	5·6	38·2	37·8	—	—
1		0·04 M-KCl added to suspension							
20		1·34	2·48	183	34·7	74·7	32·7	—	—
24		^{42}KCl added to suspension							
25		1·45	2·57	185	35·6	76·4	34·4	36·1	40·3
29	21°	Suspension warmed to 21°							
30		1·10	2·59	185	34·2	77·4	35·4	38·0	39·4
35		1·44	2·58	188	35·5	78·4	36·4	39·3	39·1
40		1·34	2·55	196	36·1	79·9	37·9	45·8	34·1
45		1·36	2·61	193	35·8	78·6	36·6	45·8	32·8
50		1·50	2·68	198	37·5	78·0	36·0	48·0	30·0
70		1·73	3·05	212	37·9	67·3	25·3	52·2	15·1
90		2·35	3·91	236	43·0	52·8	10·8	50·2	2·6

was 40·3 m-molal. This unexchanged potassium corresponded exactly with the endogenous potassium concentration of the mitochondria, after allowing for the shrinkage produced by the increased concentration of the medium. The above measurements of the distribution of solutes at 0° were similar to previous findings (Amoore & Bartley, 1958).

Effect of incubation at 21° on the distribution of solutes. At time 29 min. the remaining suspension was rapidly warmed to 21°, and incubated at this temperature for 1 hr. Samples were withdrawn and centrifuged at times 30, 35, 40, 45, 50, 70 and 90 min. from the start of the experiment. The total water content of the mitochondrial pellets was approximately constant at about 2·6 l./kg. of *M* during the first 20 min. of incubation at 21° (until time 50 min.), but thereafter the mitochondria swelled until at time 90 min. the water content of the pellet was 3·9 l./kg. of *M*, an increase of 50 %. The concentrations of sucrose and chloride in the pellets did not alter during the first 20 min. of incubation at 21°. However, accompanying the swelling of the mitochondria, there were increases in the concentrations of sucrose and chloride, until at time 90 min. both solutes had nearly the same concentrations in the pellet as in the supernatant fluid. Thus the mitochondria became completely penetrated by sucrose and chloride in 1 hr. at 21°, whereas during 8 hr. at 0° a substantial proportion of the mitochondrial volume had remained inaccessible to these solutes (Amoore & Bartley, 1958).

For the first 20 min. of incubation at 21° the total concentration of potassium in the mitochondrial pellets remained constant, but it then decreased until at time 90 min. it was only 10·8 m-molal greater than that in the medium. Thus at the same time that the above-mentioned changes in water, sucrose and chloride content took place, the mitochondria lost their ability to retain a higher concentration of potassium than that in the medium. The only change in the mitochondria observed during the first 20 min. of incubation at 21° was an increase in the radioactivity of the pellets. During the whole period of the incubation the concentration of unexchanged potassium in the mitochondrial pellets steadily decreased, until at time 90 min. only 2·6 m-molal potassium remained unexchanged.

The internal potassium of the mitochondria was diluted by the increase in water content which occurred. Therefore the results were also calculated in an alternative manner on the basis of dry weight, and expressed as solute contents (m-moles/kg. of *M*). The changes in excess of potassium and unexchanged potassium are shown graphically in Fig. 1. This confirms the conclusion that during the first 20 min. of incubation at 21° the mitochondria were able to maintain a constant concentration of potassium above that in the medium, while part of that potassium was undergoing exchange with that in the medium. During this 20 min., 22 % of the endogenous potassium content of the mitochondria exchanged with that in the medium, and at the end of an hour, 91 % had exchanged.

Extraction of lipid-bound potassium. It was considered possible that the endogenous potassium in the mitochondria might be bound by lipid materials, and that this might account for its failure to exchange at all rapidly with potassium in the aqueous medium. If that were so, the endogenous potassium should be extractable from the mitochondria with solvents for lipids. To test this, at times 25 and 90 min. (Table 1) extra samples of the mitochondrial suspension were centrifuged, the mitochondrial pellets were 'freeze-dried', and their lipid constituents were extracted. It was found that only 2·2 % of the potassium in the mitochondrial pellets was soluble in chloroform. This was far too small to account for the proportion of potassium in the pellet (53 %) which was initially unexchangeable at 0°. Indeed, the 2·2 % might represent the minute quantity of potassium extracted from its inorganic salts by chloroform under these conditions (Solomon, Lionetti & Curran, 1956). Of the total radioactivity in the mitochondrial pellets, 1·6 % was soluble in chloroform at time 25 min. and 2·5 % at 90 min.

Adsorption of potassium and magnesium by mitochondria. Although little of the mitochondrial

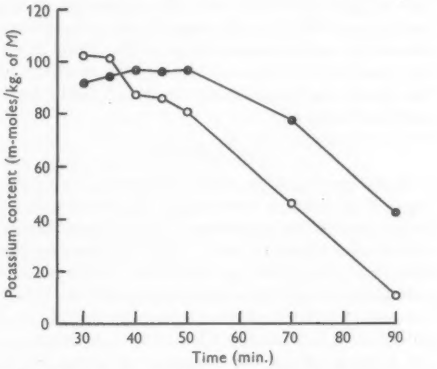

Fig. 1. Changes in potassium content of mitochondria during incubation at 21°. The data on the distribution of potassium were taken from Table 1 and recalculated on the basis of mitochondrial dry wt. (*M*), instead of water content of the pellet. ●, Potassium content of the mitochondria in excess of the concentration in the medium; ○, potassium content of the mitochondria which remained unexchanged with ^{42}K in the medium.

potassium was lipid-bound, some potassium may be bound in other ways. Amoore & Bartley (1958) showed that when potassium chloride was added to mitochondria suspended in 0·25 M-sucrose, there was a greater increase in the potassium content than in the chloride content of the mitochondria. In the experiment shown in Table 1, the difference was 11 m-moles of potassium/kg. of *M*. The following experiment suggests that this extra potassium was probably adsorbed by the mitochondria in exchange for other ions, especially H^+, in much the same way as Mn^{2+} ions are taken up (Bartley & Amoore, 1958).

The pH of a sample (2 ml.) of mitochondrial suspension in 0·25 M-sucrose at 0° was 7·17. When 0·16 ml. of 0·125 M-potassium chloride was added (which gave a concentration of 10 mM-potassium chloride in the medium) the pH decreased to 7·10. The sample was then centrifuged for analysis. Titration of a fresh sample with hydrochloric acid (see the Methods section) showed that the mitochondrial suspension had a large buffering capacity. Therefore the above small change of only 0·07 pH unit represented the production of a considerable amount of acid. It was found by analysis that the difference between the increases in potassium and chloride concentrations of the mitochondria was 7 m-moles of potassium/kg. of *M*. About two-thirds of the uptake of potassium by the mitochondria was compensated by release of H^+ ions, and the remainder by release of Mg^{2+} ions.

When magnesium chloride (final concentration 5 mM) was added to a further sample of the same suspension of mitochondria in 0·25 M-sucrose the fall in pH was 0·26 unit. The quantity of magnesium adsorbed by the mitochondria (in excess of chloride) was 26 m-equiv./kg. of *M*. Again most of the uptake was compensated by release of H^+ ions, but there was also a small loss of K^+ ions from the mitochondria.

DISCUSSION

Concentration difference of potassium. The data in Table 1 show that throughout the experiment the concentration of potassium in the mitochondrial pellet was greater than that in the medium. However, the true concentration difference was probably greater than that indicated in Table 1, because the endogenous potassium of the mitochondria was probably restricted to that phase of the mitochondrial water which was inaccessible to sucrose (Amoore & Bartley, 1958). On this assumption, the concentration of potassium within this inaccessible space was calculated to be 137 m-molal. When this calculation was applied to several different preparations of mitochondria, it showed that the concentration of potassium in the non-accessible water was in the range 101–158 m-molal. The probable concentration of potassium within mitochondria is similar to that believed to exist in the interior of liver cells (Aebi, 1953). Hence there may be no concentration difference of potassium between liver mitochondria and their surroundings *in vivo*, and therefore no requirement for active transport of potassium.

Some of the endogenous potassium may be bound by adsorption or associated with lipids. The amount extracted with a solvent for lipids was found to be less than 5% of the endogenous potassium. In the presence of 42·0 m-molal potassium chloride, the mitochondria took up potassium in excess of chloride. The amount so taken up (probably by adsorption in exchange for H^+ and Mg^{2+} ions) was equivalent to only about 10% of the quantity of endogenous potassium. It was not possible to estimate how much of the endogenous potassium was bound to adsorption sites within the mitochondria, but competition with endogenous Mg^{2+} ions would tend to decrease the binding of endogenous potassium. However, indirect evidence that most of the endogenous potassium of mitochondria is in free solution is provided by the fact that the non-accessible volume of isolated mitochondria shows an osmotic responsiveness (Amoore & Bartley, 1958). Analysis has shown no endogenous solutes other than the potassium phosphates which could account quantitatively for the internal osmotic pressure of the mitochondria. Gamble (1957) suggested that all the potassium retained by isolated mitochondria is tightly bound to acceptor sites on the structural elements of the mitochondria. However, apart from the above considerations, this seems unlikely in view of Gamble's finding that only about one-tenth of the mitochondrial potassium remained associated with the fragments obtained after disrupting the mitochondria with digitonin.

These considerations suggest that the endogenous potassium of isolated mitochondria is largely in free solution in the non-accessible phase of the mitochondrial water, and that its concentration is probably over 100 m-molal. Therefore a true concentration difference of at least 60 m-molal potassium may have been maintained between mitochondria and medium during the first 20 min. of incubation at 21° (Table 1).

Permeability, adsorbency and volume of mitochondria. During the first 20 min. of incubation at 21° (Table 1) there were no changes in the water content of the mitochondrial pellets obtained on centrifuging. Therefore the volume of the mitochondria was constant during this period. The sucrose and chloride contents of the pellets were also constant, showing that the permeability of the mitochondria towards these two solutes did not alter. Nor did the potassium content of the pellets

vary, showing that there was no overall change in the adsorption of potassium by the mitochondria, or in the permeability of the mitochondria to potassium, i.e. there was no net flux of K^+ ions. Furthermore, the mitochondria maintained a concentration of potassium at least 35 m-molal higher than that in the medium. During this 20 min., 22% of the endogenous potassium of the mitochondria exchanged with labelled potassium from the medium. Hence, during this period, while the permeability, adsorbency and volume of the mitochondria were substantially constant, an exchange of K^+ ions took place, across a concentration difference of potassium. Ignoring for the moment the question of any electrical-potential difference, and considering only the chemical-concentration difference (see introductory paragraphs and papers there cited), it is possible to argue that during this period the exchange of K^+ ions was maintained by an active process.

Stanbury & Mudge (1953), Mudge (1955) and Gamble (1957) have also measured the exchange of ^{42}K between medium and mitochondria. However, they did not establish that a concentration difference of potassium was maintained between mitochondria and medium during the incubation. Nor did they obtain enough data to show that the results were not due to changes of mitochondrial volume, permeability or adsorbency. Instead they demonstrated that once the mitochondria had become labelled with ^{42}K, the radioactivity could not be removed by washing at 0° with solutions of sucrose, sodium chloride or potassium chloride. This provides valuable evidence (not obtained in the present experiment) that the ^{42}K had passed through a permeability barrier.

Effect of temperature. Although the results in Table 1 (at 21°) are not strictly comparable with those obtained at 0° (Amoore & Bartley, 1958), because of the different media, the rate of exchange of the endogenous potassium was about ten times as fast at the higher temperature (compare Mudge, 1955). A temperature coefficient (Q_{10}) of over 3 suggests either the presence of high potential-energy barriers (Danielli & Davson, 1935) or the participation of a metabolic process. Stanbury & Mudge (1953), Mudge (1955) and Gamble (1957) have demonstrated that the rate of exchange of mitochondrial potassium is closely related to the metabolic activity of the particles.

Swelling of mitochondria. In the experiment shown in Table 1, the mitochondria began to swell after 20 min. of incubation at 21°, became more permeable to sucrose and chloride and progressively lost their ability to maintain an excess concentration of potassium. Although an exchange of potassium continued, it was no longer possible to argue that active transport was taking place. The exchange could have been due to the breakdown of the permeability barriers enclosing the previously inaccessible space, thus permitting the loss of endogenous potassium, and the entry of sucrose, chloride and labelled potassium from the medium.

When the mitochondria swelled (Table 1) it was the space already accessible to exogenous sucrose and chloride which swelled, and not the space which was initially inaccessible; the inaccessible space actually decreased in volume. Experiments described previously also show that when mitochondria swell, solutes from the medium enter the swollen volume (Bartley & Amoore, 1958; Amoore, 1958). These findings suggest that the swelling cannot be due to any internal osmotic pressure developed by crystalloids. The swelling of mitochondria, which occurs in certain unfavourable conditions, bears a marked resemblance to the so-called 'non-osmotic haemolysis' of erythrocytes described by Wilbrandt (1941). He considered that this form of haemolysis was caused by 'colloid osmotic pressure' developed by soluble protein within the erythrocyte. About half the protein of mitochondria is released in soluble form on disrupting the particles (Hogeboom & Schneider, 1950; Spector, 1953). Furthermore, the swelling of mitochondria can be prevented or reversed by adding colloids to the medium, whereas sucrose is ineffective (Lehninger, Ray & Schneider, 1959). Therefore isolated mitochondria may tend to swell if their limiting membranes become permeable to sucrose and salts while remaining impermeable to colloids.

Possible active transport. The experiment described above demonstrates that a preparation of isolated mitochondria is able to perform an exchange of K^+ ions across a concentration difference of potassium. Whereas it is basically similar to experiments described by other authors (see introductory paragraphs), it is felt that the modified technique meets the criticisms to which the earlier experiments might be subjected.

Nevertheless it has still not been proved that mitochondria are capable of active transport, because there is no knowledge of the potential difference across the mitochondrial membrane. The distribution of other ions (not estimated in this experiment) may have been such as to establish a potential difference of the appropriate sign and magnitude, so that the K^+ ions on each side of the membrane were actually in equilibrium, even though having different concentrations. The lower concentration of Cl^- ions inside the mitochondria than in the medium suggests that there may be a potential difference of the correct sign. The apparent concentrations of K^+ ions and Cl^- ions, in the phase of the mitochondrial water which was inaccessible to sucrose, were calculated for the

pellet obtained at time 25 min. from the start of the experiment. This calculation gave 180 m-molal K^+ ions and 17 m-molal Cl^- ions in the inaccessible space, compared with 42 m-molal potassium chloride in the medium. The concentration ratios were as follows:

$$\frac{K^+_{inside}}{K^+_{outside}} = 4{\cdot}3. \quad \frac{Cl^-_{outside}}{Cl^-_{inside}} = 2{\cdot}5.$$

These ratios are sufficiently close to each other, in view of the possibilities of error in estimation, to suggest that the distribution of K^+ and Cl^- ions might be determined by a Gibbs–Donnan equilibrium. Thus the K^+ and Cl^- ions may have been at equal electrochemical potential, and their distributions were not necessarily maintained by an active process.

The unequal distribution of K^+ and Cl^- ions may have been maintained by the presence of a high concentration of relatively indiffusible, negatively charged ions within the mitochondria. Werkheiser & Bartley (1957) showed that isolated mitochondria contain phosphate approximately equivalent, on a molar basis, to the potassium they contain, and that about 84% of this phosphate is esterified. The mitochondrial membrane is considerably less permeable to adenosine 5′-phosphate and to adenosine triphosphate than to Na^+ ions (Amoore, 1958). The permeabilities to Na^+ and K^+ ions are similar (Amoore & Bartley, 1958). Hence it is probable that the endogenous organic phosphates of the mitochondria are indeed less capable of penetrating the mitochondrial membrane than are the K^+ ions. These findings favour the idea that the organic phosphates retained within the mitochondria may be able to support a large difference between internal and external concentrations of K^+ ions. In these circumstances an exchange of K^+ ions could take place, across a concentration difference of K^+ ions, and without any requirement for metabolic energy. Passage of the ions through the permeability barrier offered by the mitochondrial membrane demands only a sufficiency of potential energy (thermal energy), and does not require work to be done (Danielli & Davson, 1935).

Organic phosphate escapes from isolated mitochondria, during prolonged storage at 0°, or in response to treatment with orthophosphate (Amoore & Bartley, 1958; Amoore, 1958). This loss of organic phosphate is accompanied by a loss of K^+ ions as well. Presumably the mitochondrial membrane becomes permeable to the organic phosphate, which escapes, with the result that the mitochondria can no longer maintain a concentration of K^+ ions in excess of that in the medium. In the present experiment (Table 1), the net efflux of K^+ ions from the mitochondria, after the first 20 min. of incubation at 21°, may have been due to the mitochondrial membrane becoming permeable to the endogenous organic phosphates.

SUMMARY

1. Isolated rat-liver mitochondria were incubated for 60 min. at 21° in 0·25 M-sucrose plus 0·04 M-potassium chloride containing ^{42}K. At intervals, samples of the suspension were centrifuged for analysis of the sedimented pellet and supernatant fluid. During the first 20 min. of incubation, the mitochondria maintained an internal concentration of potassium at least 35 m-molal higher than that in the medium. There were no changes in the permeability, adsorbency or volume of the mitochondria. In this time, 22% of the endogenous potassium of the mitochondria exchanged with labelled potassium from the medium.

2. The exchange of potassium may have been due to active transport of K^+ ions into the mitochondria, but the participation of an active process was not definitely proved.

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REFERENCES

Aebi, H. (1953). *Helv. physiol. acta*, **11**, 96.
Amoore, J. E. (1958). *Biochem. J.* **70**, 718.
Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
Bartley, W. & Amoore, J. E. (1958). *Biochem. J.* **69**, 348.
Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
Danielli, J. F. & Davson, H. (1935). *J. cell. comp. Physiol.* **5**, 495.
Gamble, J. L. (1957). *J. biol. Chem.* **228**, 955.
Hogeboom, G. H. & Schneider, W. C. (1950). *Nature, Lond.*, **166**, 302.
Lehninger, A. L., Ray, B. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 97.
Macfarlane, M. G. & Spencer, A. G. (1953). *Biochem. J.* **54**, 569.
Mudge, G. H. (1955). In *Electrolytes in Biological Systems*, p. 112. Ed. by Shanes, A. M. Washington, D. C.: American Physiological Society.
Price, C. A., Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 754.
Rosenberg, T. (1954). *Symp. Soc. exp. Biol.* **8**, 27.
Solomon, A. K., Lionetti, F. & Curran, P. F. (1956). *Nature, Lond.*, **178**, 582.
Spector, W. G. (1953). *Proc. Roy. Soc.* B, **141**, 268.
Stanbury, S. W. & Mudge, G. H. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 675.
Ussing, H. H. (1949). *Physiol. Rev.* **29**, 127.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
Wilbrandt, W. (1941). *Pflüg. Arch. ges. Physiol.* **245**, 22.

The Biochemistry of Rumen Protozoa

3. THE CARBOHYDRATE METABOLISM OF *ENTODINIUM**

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The rumens of sheep and cattle commonly contain ciliate Protozoa belonging to numerous genera. The biochemical study of these organisms has been hampered in the past by the difficulty of separating the different types from bacteria, and from one another, in sufficient quantity. The conventional bacterial pure-culture techniques have not so far proved applicable to these organisms, and investigators have therefore had to rely mainly upon alterations to the ruminant's diet, and various manipulations of the rumen liquor *in vitro*, in attempts to obtain suspensions of individual types of Protozoa. A technique of considerable value in this field was originated by Eadie & Oxford (1957), who found that many species of Protozoa could be eliminated from the sheep rumen by emptying and thorough washing of that organ. In a number of sheep treated in this way, only the smaller species of the oligotrich ciliate *Entodinium* survived the emptying procedure. It was further found (J. M. Eadie, personal communication, 1958) that when the diet of a treated sheep was altered by including a moderate amount of starchy foods, the numbers of entodinia present in the rumen increased to such an extent that suspensions of mixed *Entodinium* species, free from other Protozoa, and almost free from bacteria, could readily be prepared from the rumen liquor. Because *Entodinium* can, under many feeding régimes, be the predominant protozoal genus in the rumen, and because the biochemistry of these organisms has not been studied previously, the present investigation was undertaken. Some of our observations have already been reported briefly (Abou Akkada, Hobson & Howard, 1959).

MATERIALS AND METHODS

Sheep as source of entodinia. The single sheep (no. 105) which served throughout these experiments had previously had its rumen fauna partially removed by the method of Eadie & Oxford (1957), and thereafter was kept isolated from other ruminants. Before the present work was commenced it had been inoculated with *Dasytricha ruminantium*, but during our experiments only very small numbers of this organism were present. In addition to a feed of hay (450 g.) morning and afternoon, the sheep was offered daily 450 g. of a concentrates ration, approximately three-quarters of which consisted of ground maize and crushed oats.

Preparation of protozoal suspensions. Samples of rumen liquor were withdrawn 3 hr. after the concentrates meal, strained through gauze and mixed in a separating funnel with an equal volume of the bicarbonate buffer solution described below (cf. Oxford, 1958). All manipulations and incubation of protozoa were carried out at 38°. During incubation for 1 hr. the entodinia settled to the bottom of the funnel as a grey–white layer, which was run off into boiling tubes containing the buffer solution. From this stage onwards the buffer solutions used always contained chloramphenicol (50 μg./ml.) to suppress bacteria. After threefold washing by decantation, the protozoa formed a clean white layer in the bottom of the tubes. Microscopic examination of the protozoa, both living and after staining with iodine, showed them to be very active and filled with grains of plant starch at this stage. The small numbers of *D. ruminantium* in the suspensions were killed by incubating overnight in buffer solution containing 0·6% of mannose. After washing out the mannose by three changes of buffer solution the suspensions consisted almost wholly of *Entodinium caudatum*, with a very few small organisms similar to *Entodinium simplex*. Neither chloramphenicol nor mannose appeared to harm the entodinia. The suspensions could be kept alive for 3 days by changing the buffer–chloramphenicol solution every 12 hr. At the end of this period the cells were almost empty of starch.

Buffer solutions. The solutions used were based on that of Coleman (1958). The one normally used contained (in 1 l.); K_2HPO_4, 6·3 g.; KH_2PO_4, 5·0 g.; $NaHCO_3$, 1 g.; NaCl, 0·6 g.; $MgSO_4,7H_2O$, 0·1 g.; $CaCl_2,6H_2O$, 0·09 g.; Na_2S, $9H_2O$, 0·2 g. The pH was 6·9–7·0. In experiments which required the analysis of fermentation gases, the $NaHCO_3$ was replaced by sodium acetate (0·75 g./l.).

Materials. These were the same as those used by Howard (1959*a*, *b*). Amylose, amylopectin, β-dextrin and maltotriose were given by Dr P. N. Hobson. Chloramphenicol was B.P. quality, manufactured by Parke, Davis and Co. Ltd., Hounslow.

Bacterial examination of protozoal suspensions. Portions of protozoal suspensions were inoculated into the semi-solid medium of Hobson, Mann & Oxford (1958), in which soluble starch replaced the sodium lactate.

Fermentation procedures. (*a*) Endogenous fermentation. Sufficient washed protozoa were placed in 100 ml. conical flasks to just cover the bottom. This usually required the protozoa from 250 ml. of rumen liquor/flask. The flasks

* Part 2: Howard (1959*b*).

were immediately filled almost to the top with buffer–chloramphenicol solution, gassed for 3 min. with $N_2 + CO_2$ (95:5) and sealed with rubber stoppers carrying Bunsen valves. Samples for analysis were removed at intervals during incubation, after mixing the contents of the flasks by gentle swirling.

(*b*) Incubation with soluble sugars. A suspension of protozoa which had been starved for 36 hr. was divided equally between two flasks, which were treated as described above, except that to one flask the substrate was added to a final concentration of 0·2%. For analysis the samples of suspension were centrifuged, and the carbohydrate in the supernatant was determined by the anthrone method (Trevelyan & Harrison, 1952).

(*c*) Analysis of gases formed during endogenous fermentation. A suspension (200 ml.) of washed entodinia in the acetate buffer–chloramphenicol was placed in a 500 ml. flask immersed in a water bath at 38°. A slowly flowing stream of N_2, which had previously been bubbled successively through sodium pyrogallate and water, was delivered below the surface of the suspension. The determination of CO_2, H_2 and hydrocarbons in the emergent gases was carried out as described by Heald & Oxford (1953), except that the addition of O_2 to the gases before passing over the heated CuO was omitted. The fermentation was allowed to proceed for 6 hr., after which time 4N-H_2SO_4 (2 ml.) was added to the suspension to kill the protozoa and release bound CO_2. The gas flow was continued for 1 hr. after addition of the acid. The weight of CO_2 found in the absorption tube was corrected for the small amount found in a blank experiment when buffer solution without protozoa was used.

Manometric methods. The methods for measuring the effect of protozoa on added carbohydrates were as described by Howard (1959*a*). In other experiments samples were taken from a suspension of entodinia at intervals during a 48 hr. incubation period and tested for rate of endogenous fermentation. The gaseous products of fermentation were also measured manometrically, by the method of Elsden & Lewis (1953). Warburg double-side-arm cups were used, containing protozoal suspension in acetate buffer (2 ml.) under an atmosphere of N_2, with 2N-NaOH (0·2 ml.) absorbed in filter paper in one side arm, and 4N-H_2SO_4 (0·4 ml.) in the other.

Preparation of cell-free extracts. Washed protozoa, freed from as much supernatant buffer solution as possible, were frozen and stored at −20°. When required, the frozen mass was thawed by incubating at 38° for 1 hr. and the suspension was mixed in a mortar with 3 vol. of carborundum powder (4 F mesh, Griffin and George Ltd., Wembley, Middlesex). The stiff paste was ground by hand for 1 hr., transferred with a little water to centrifuge tubes and centrifuged at 20 000 *g* for 30 min. at 0°. After dialysing and centrifuging again (Howard, 1959*b*), the supernatant yielded a clear solution, which was stored at 1° under toluene. About 15 ml. of solution, containing approx. 180 μg. of protein N/ml., was obtained from the entodinia from 1 l. of rumen liquor.

Tests for carbohydrase activity. Qualitative tests were made as described by Howard (1959*b*). In addition, a chromatographic solvent, consisting of the top layer of benzene–pyridine–butanol–water mixture (1:3:5:3, by vol.) was used in examining amylose hydrolysates for maltotriose. In quantitative experiments with amylase, the procedure of Hobson & MacPherson (1952) was followed. To measure maltase activity, tubes containing maltose (100 μg.), 0·1 M-sodium citrate buffer (0·3 ml.) and suitably diluted enzyme (0·5 ml.) in a total volume of 1 ml. were placed in a water bath at 38° for 3 hr. The reducing power of the mixtures was measured by Nelson's (1944) method, and the extent of hydrolysis of maltose was calculated from the reducing power of maltose and glucose determined at the same time.

The separation of the amylase and maltase activities in the extracts was attempted by the method of Hockenhull & Herbert (1945). An ice-cold mixture of enzyme solution (5 ml.), Na_2SO_4 (50 mg.) and ethanol (5 ml.) was shaken for 2 hr. with potato starch [1 ml. of a 12·5% (w/v) suspension in 50% ethanol]. The starch was subsequently washed twice with 10 ml. of 0·067 M-phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 5·8. The aqueous ethanol and phosphate solutions were dialysed and their amylase and maltase activities were determined.

Analytical methods. The starch in the protozoal cells was determined by an adaptation of the methods of Pirt & Whelan (1951) and Trevelyan & Harrison (1952). A portion (2 ml.) of the well-mixed suspension of protozoa was centrifuged at 600 *g* for 20 min. and the clear supernatant was decanted. In cases where soluble sugars had been added to the suspension, the cells at this stage were washed once with water (1 ml.). The cells were suspended in 0·25N-NaOH (4 ml.) and the tubes were heated in boiling water for 3 min. After being centrifuged as before, a portion of the clear supernatant was suitably diluted and the total glucose present was determined by the anthrone method. Chromatographic examination of an acid hydrolysate (2N-H_2SO_4 for 1 hr. at 100°) of the alkaline extract showed that no sugars other than glucose were present.

Volatile fatty acids, except formic acid, were detected on paper chromatograms by the method of Elsden & Lewis (1953); formic acid was detected by the ninhydrin method of Burness & King (1958). Quantitative determination of volatile fatty acids, lactic acid, total N and protein N was carried out as described by Howard (1959*a*). Dry matter in the suspensions was determined by drying them for 5 hr. at 105°, and correcting the weight of residue for the weight of salts remaining after drying an equal volume of buffer solution.

RESULTS

As prepared for use in fermentation experiments, the suspensions consisted almost wholly of *E. caudatum*, actively motile and well filled with grains of plant starch. In three of these suspensions, the protozoal dry matter was 11, 20 and 31 mg./ml. respectively. The protein content (total N × 6·25) was 23–26% (mean 25%) and the starch content (as glucose) 59–68% (mean 64%) of the protozoal dry matter. No bacteria able to grow in the semi-solid starch medium were ever observed when inoculations were made from the chloramphenicol-treated suspensions.

Endogenous fermentation by entodinia. When the suspensions were incubated in the bicarbonate buffer–chloramphenicol the organisms remained active for more than 48 hr., during which time the

starch in the cells largely disappeared, as judged by the iodine reaction. These microscopic observations accord with the quantitative results shown in Fig. 1. During incubation for 24 and 48 hr., 61 and 92% respectively of the starch originally present disappeared. The dwindling metabolic activity of the protozoa in the aging suspension, suggested by the shape of the curve in Fig. 1, is also illustrated by the manometric results (Fig. 2). Gas production by suspensions of all ages tested was linear during the first 30 min. of the manometric experiments. The rate of gas production, expressed as μl. of carbon dioxide/hr./0·1 mg. of N, dropped by almost a half during the first 24 hr. in the life of a suspension. This rate seemed remarkably constant between suspensions prepared at different times.

The products formed by the entodinia during the fermentation of ingested starch included volatile fatty acids, lactic acid, carbon dioxide and hydrogen. It was not possible to determine all of these products in the same suspension; the gaseous products on the one hand, and the volatile fatty acids and lactic acid on the other, had to be determined in separate experiments, as shown in Table 1. The volatile fatty acids, upon chromatographic analysis, were shown to consist of formic acid, acetic acid, propionic acid and butyric acid only, with no trace of any higher acid (Table 2). The acetic acid and butyric acid together accounted for 82 and 89% of the total volatile fatty acids in Expts. 1 and 2 respectively.

Effect of entodinia on added substrates. The gas production during incubation of 24 hr. old suspensions in the Warburg apparatus with glucose, maltose or rice-starch grains for 1 hr. was identical with that of control portions of suspensions incubated without added substrates. Microscopic examination of iodine-stained drops of the suspensions at the end of the run suggested that no conversion of soluble sugar into storage starch had occurred. Most of the starch grains, however, had been swallowed by the protozoa. In other experiments duplicate portions of protozoal suspensions were incubated with and without added soluble carbohydrate. Frequent samples were taken, and the starch content of the cells and the carbohydrate content of the solution were measured. The results of an experiment with glucose are shown in Fig. 3; similar results were obtained by the use of maltose, cellobiose, sucrose and soluble starch.

To a suspension of protozoa 36 hr. old, granular rice starch was added (4 mg./ml.), and the incubation continued for 12 hr. more. The endogenous fermentation rate, measured manometrically, had then risen above the value found just before addition of the starch (Fig. 2).

Carbohydrases in cell-free extracts of entodinia. The extracts of the protozoal cells had no

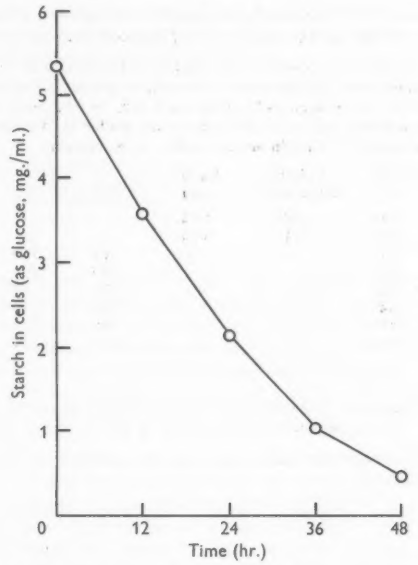

Fig. 1. Fermentation of ingested starch by suspensions of *E. caudatum*. For conditions of incubation see text.

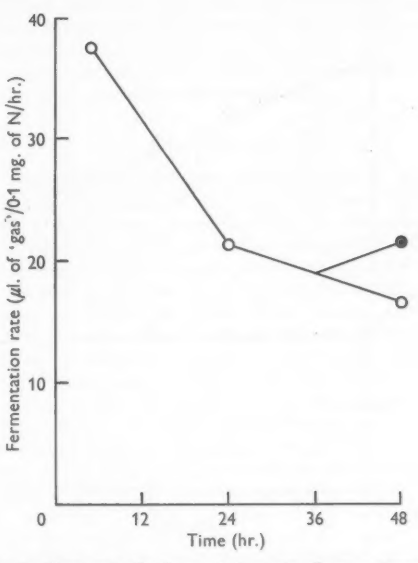

Fig. 2. Changes in the fermentation rate of suspensions of *E. caudatum* with age. ○, Fermentation rate of starved protozoa; ●, fermentation rate 12 hr. after adding granular rice starch (0·2%). For conditions see text.

Table 1. *Products of fermentation of ingested plant starch grains by suspensions of* Entodinium *species*

Results are expressed as μmoles of product from the fermentation of 100 μmoles of starch, as glucose. Experimental conditions: A1, 48 hr. and A2, 24 hr., both in bicarbonate buffer; B, 1 hr. in acetate buffer in Warburg apparatus; C, 6 hr. in acetate buffer, in N_2 stream.

Expt. no.	Volatile fatty acids	Lactic acid	CO_2	H_2
A1	156	4·42	—	—
A2	141	3·70	—	—
B1	—	—	107	100
B2	—	—	98	100
B3	—	—	104	83
B4	—	—	103	93
C1	—	—	120	122
C2	—	—	125	137

Table 2. *Volatile fatty acids produced in starch fermentation by suspensions of* Entodinium

Results are expressed as molar percentage of total volatile fatty acids.

Expt. no.	Formic acid	Acetic acid	Propionic acid	Butyric acid
A1	5	35	13·5	46·5
A2	4	34	7	55

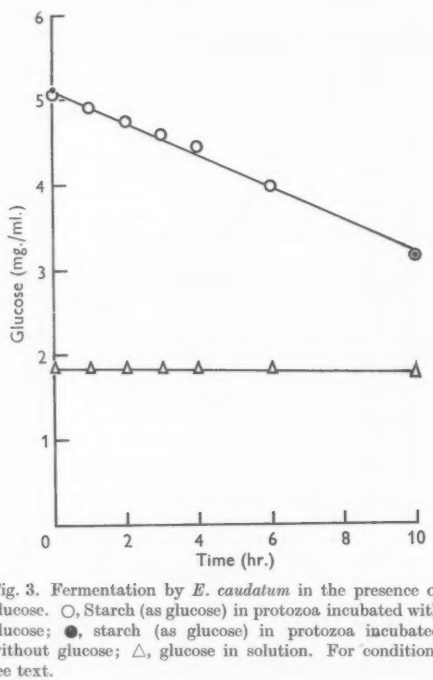

Fig. 3. Fermentation by *E. caudatum* in the presence of glucose. ○, Starch (as glucose) in protozoa incubated with glucose; ●, starch (as glucose) in protozoa incubated without glucose; △, glucose in solution. For conditions see text.

chromatographically detectable effect on lactose, raffinose, xylobiose and wheat-flour pentosan (Howard, 1957). Very slight hydrolysis of cellobiose, trehalose, melibiose, α-methyl glucoside, sucrose, inulin and bacterial levan was observed, but significant hydrolysis occurred of amylose, amylopectin, glycogen, β-dextrin and maltose only. Glucose and maltose were the main products from the polysaccharides, with smaller amounts of a substance chromatographically indistinguishable from maltotriose. With the decrease of the 'blue value' of amylose during incubation for 30 min. as a measure of activity, the amylase in the extracts was tested under different conditions. The variations of activity with pH and temperature are shown in Figs. 4 and 5 respectively. The optimum pH at 38° was 5·5, and the optimum temperature at pH 7 was 38°. The amylase appears to be unstable at 45°, since the activity at this temperature during incubation for 1 hr. was less in proportion to activity at the optimum temperature, than during 30 min. In 0·2 M-sodium citrate buffer, pH 7, the amylase activity was unchanged by the addition of NaCl to a final concentration of 0·14 M. In 0·2 M-sodium acetate buffer, pH 6·9, the amylase activity was decreased by about 23 and 40 % when $CaCl_2$ was present in concentrations of 0·017 and 0·07 M respectively. When the cell

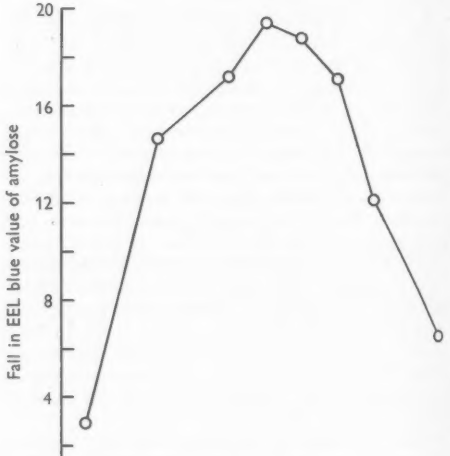

Fig. 4. Variation of activity of *E. caudatum* amylase with pH. Reactions were studied in 0·1 M-sodium citrate buffers of pH indicated. For other conditions see text.

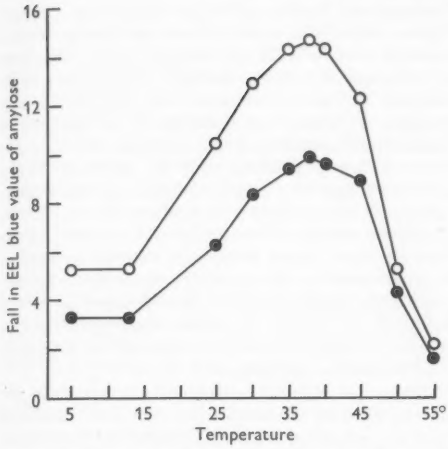

Fig. 5. Variation of activity of *E. caudatum* amylase with temperature. ●, Activity during incubation for 30 min.; ○, activity during incubation for 60 min. For conditions see text.

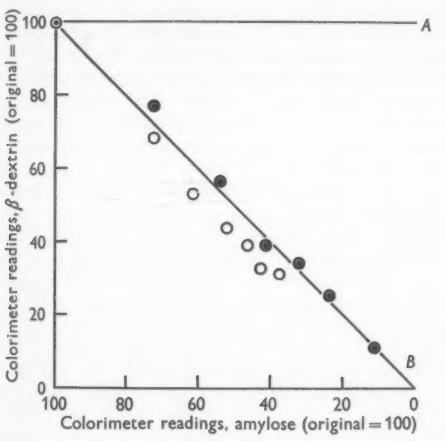

Fig. 6. Comparison of the action of salivary amylase and *E. caudatum* amylase on amylose and β-dextrin. *A*, Theoretical curve for β-amylase; *B*, theoretical curve for α-amylase. ●, *E. caudatum* amylase; ○, human salivary amylase. Amylose digests contained 1 mg. of amylose/ml. Samples (1 ml.) were diluted with iodine solution (I_2, 2 mg.; KI, 20 mg., in 100 ml. of water) and readings obtained in an EEL colorimeter (Evans Electroselenium Ltd.) with an Ilford no. 608 filter. β-Dextrin digests contained 1 mg. of β-dextrin/ml. Samples (2 ml.) were diluted with iodine solution (I_2, 4 mg.; KI, 40 mg., in 100 ml. of water) and readings obtained with an Ilford no. 626 filter. All digests were incubated at 38°.

Table 3. *Fractionation of amylase and maltase in a cell-free extract of* Entodinium *by the method of Hockenhull & Herbert* (1945)

Fraction	Activity (% of original) Amylase	Maltase
Crude extract	100	100
Ethanol solution after starch treatment	37	45
Phosphate extract	59	0
Recovery	96	45

extract was allowed to act upon amylose and β-dextrin simultaneously, and the effects on the two substrates were calculated and plotted as described by Hobson & MacPherson (1952), the graph shown in Fig. 6 was obtained. A similar experiment, in which centrifuged and diluted human saliva was used, gave similar results, which are also shown in Fig. 6.

The maltase in the protozoal extracts had maximum activity at pH 5 in the citrate buffer.

Treatment of a cell-free extract according to the method of Hockenhull & Herbert (1945) yielded two fractions, one an ethanolic solution containing material not adsorbed on to the potato starch, and another containing material originally adsorbed on to the starch, and extractable into phosphate buffer at pH 5·8. The amylase and maltase activities of these solutions, compared with the activities of the crude extract, are shown in Table 3. When the purified amylase acted upon amylose the main product was maltose; a little maltotriose and a trace of glucose were also formed.

DISCUSSION

The genus *Entodinium* Stein contains a large number of species, all of which have been found only in the rumen. In addition to the differentiation of these species on anatomical grounds, the entodinia can also be grouped physiologically by their feeding habits, according to the materials observed within the cells. Thus Bhatia (1936) mentions other ciliates, flagellates, bacteria, pollen grains, 'vegetable particles' and 'small plant débris' as forming the food of different *Entodinium* species. Clearly the genus is very heterogeneous biochemically, and little useful information would be obtained from suspensions of mixed *Entodinium* species. We have been fortunate in that the preparations we have used consisted very largely of *E. caudatum*. It has been known for some time from microscopic observations that this species is primarily a starch feeder (Hungate, 1943), whereas the minor constituents of the suspensions, those Protozoa resembling *E. simplex*, are most probably not. We consider that the properties of the sus-

pensions used in this work can be taken to be the properties of *E. caudatum* alone.

The present biochemical observations suggest that *E. caudatum* satisfies its carbohydrate requirements wholly by the ingestion and fermentation of starch grains, and not by any other means. Thus none of the soluble sugars tested, nor a solution of soluble starch, was fermented during incubation for 10 hr., nor did these substances affect the course of the endogenous fermentation of the starch stored within the cells.

The products formed during starch fermentation by *E. caudatum* resemble qualitatively those formed by numerous other carbohydrate-fermenting bacteria and Protozoa of the rumen, namely, volatile fatty acids, lactic acid, carbon dioxide and hydrogen. In making a quantitative determination of fermentation products, a difficulty was encountered which we were unable to overcome. To measure the production of carbon dioxide and volatile fatty acids by the same suspension of protozoa would require a buffer solution containing neither bicarbonate nor acetate. Such solutions, buffered only by phosphate, were invariably harmful to *Entodinium* suspensions, so that the protozoa were dying after incubation for 6 hr. Simultaneous measurement of carbon dioxide and volatile acid production has therefore proved impossible so far. The buffer solution containing acetate maintained the entodinia in a healthy state long enough for a short-term (6 hr.) experiment to be carried out, in which the fermentation gases were analysed. These gases were also analysed in 1 hr. experiments in the Warburg apparatus; these results suggest a relatively slightly lesser conversion of starch into gases during the earlier part of the fermentation period (Table 1). The lactic acid and volatile fatty acids were measured in other experiments, with the bicarbonate buffer; a longer incubation period (24 or 48 hr.) was required here, in order to obtain enough volatile acids for analysis. It is clear that a true carbon balance and hydrogen:oxygen ratio cannot be calculated with certainty from our results. If, however, it is assumed that the form of starch fermentation by *E. caudatum* is the same over different periods, and in different buffer solutions, apparent carbon balances and hydrogen:oxygen ratios may be calculated. When this is done, the carbon recovery appears to be 96 % of the carbon in the starch fermented, and the hydrogen:oxygen ratio in the products appears to be 2·1. Although a great deal of reliance cannot be placed in these two figures, we believe they show that no major product of starch fermentation has been overlooked in our analyses.

The most striking feature of the results is the low proportion of starch converted into lactic acid by *E. caudatum*, when compared with other ruminal starch-fermenting organisms. The distribution of carbon in the products is shown in Table 4, with the corresponding figures for endogenous fermentation of storage starch by rumen holotrichs for comparison. The products of fermentation of added glucose by the holotrichs may also justifiably be included here. An important starch-decomposing bacterium in the rumen, *Streptococcus bovis*, produces lactic acid and volatile acids, but no carbon dioxide. The proportions of lactic acid and volatile acids vary widely with conditions of culture (Dr P. N. Hobson, personal communication), so that it is not possible to include figures for this organism also in Table 4. The low rate of lactic acid production characteristic of *E. caudatum* could be of value to an animal whose rumen contains a high proportion of this organism. The sudden ingestion of large amounts of starch can be harmful to ruminants, because of the rapid production of large amounts of lactic acid, and the consequent fall of pH of the rumen contents to an abnormally low level (Scarisbrick, 1954). The presence of micro-organisms which can not only store away added starch for a more leisurely digestion, but can also, during the course of this digestion, produce insignificant amounts of lactic acid, would clearly be of benefit to the ruminant in preventing the development of this undesirable acidity.

A surprising result of our manometric experiments is that, although starved entodinia readily swallow rice-starch grains, no increase in fermentative activity is seen, at least during the first hour. Only after a longer period is an increase found (Fig. 2). This contrasts with the observation that *Isotricha* suspensions increase their rate of fermentation immediately on adding rice starch (Howard, 1959*a*).

Table 4. *Distribution of carbon in the products of fermentation of starch or glucose by rumen Protozoa*

Results are expressed as percentage of carbon in total carbon of the fermentation products.

Organism and substrate	Products		
	Lactic acid	Volatile fatty acids	CO_2
Isotricha + *Dasytricha* storage starch*	53	32	15
Isotricha + *Dasytricha* added glucose†	38	44	18
Dasytricha added glucose‡	48	31	22
Entodinium caudatum ingested starch§	2	80	19

* Calculated from table 4 of Heald & Oxford (1953).
† Calculated from table 2 of Heald & Oxford (1953).
‡ Calculated from table 1 of Gutierrez (1955).
§ Present results.

In the cell-free extracts made from the *Entodinium* suspensions, the only carbohydrase activities found in significant concentration were amylase and maltase. This finding agrees with the previous conclusion that *E. caudatum* relies solely on starch for satisfying its carbohydrate needs. The removal of all the maltase activity from the amylase, together with the chromatographic evidence and the effect of enzyme action on the iodine-staining properties of amylose, amylopectin and β-dextrin, show the enzyme to be a fairly typical α-amylase. The *E. caudatum* α-amylase is not identical with any of the α-amylases already investigated in rumen micro-organisms. Those of the holotrichs *Dasytricha* and *Isotricha* examined by Mould & Thomas (1958) have optimum pH values of 5·0 and 6·0 (*Dasytricha*) and 4·8 (*Isotricha*). The α-amylase of the rumen oligotrich *Epidinium caudatum* (Bailey, 1958) has maximum activity at pH 6, and an optimum temperature range of 37–45°. *S. bovis* α-amylase has maximum activity at pH 5·5–6·5, and an optimum temperature of 48° (Hobson & MacPherson, 1952). The distinction between this latter enzyme and the *Entodinium* amylase is particularly important. Gutierrez & Davis (1959) have demonstrated the ingestion by species of *Entodinium*, including *E. caudatum*, of large numbers of bacteria, identified by subsequent culture as *S. bovis*. The differences between the amylase in the *Entodinium* preparations and that of *S. bovis* show that the enzyme is a truly protozoal product, not a bacterial one.

SUMMARY

1. Active suspensions, consisting almost wholly of *Entodinium caudatum*, and free from bacteria, have been obtained from the rumen liquor of a sheep.

2. The protozoal cells in freshly prepared suspensions were filled with grains of plant starch, which gradually disappeared during incubation. The principal products formed during the fermentation were volatile fatty acids (chiefly butyric acid and acetic acid, with a little propionic acid and formic acid), carbon dioxide, hydrogen and a little lactic acid.

3. The *E. caudatum* suspensions did not metabolize sugars, or soluble starch, given in solution.

4. *E. caudatum* readily swallowed grains of rice starch, but this did not cause an immediate rise in the fermentation rate.

5. Cell-free extracts were prepared by grinding the suspensions with carborundum. Strong maltase and amylase activity was found in the extracts, but only traces of other carbohydrases. The amylase was examined in detail, and compared with similar enzymes in other rumen protozoa. It is an α-amylase.

6. It is concluded that *E. caudatum* satisfies its carbohydrate requirements solely by the ingestion of granular starch.

We are greatly indebted to our colleague, Dr J. M. Eadie, for placing the sheep at our disposal, and for her advice on the Protozoa; also to our colleague, Dr P. N. Hobson, for the gift of starch materials and for advice on a number of aspects of this work. We thank Mrs M. G. Campbell, Miss C. M. Davidson and Mr W. Shand for their assistance.

REFERENCES

Abou Akkada, A. R., Hobson, P. N. & Howard, B. H. (1959). *Biochem. J.* **73**, 44P.
Bailey, R. W. (1958). *N.Z. J. agric. Res.* **1**, 825.
Bhatia, B. L. (1936). *The Fauna of British India* (Protozoa: Ciliophora). London: Taylor and Francis Ltd.
Burness, A. T. H. & King, H. K. (1958). *Biochem. J.* **68**, 32P.
Coleman, G. S. (1958). *Nature, Lond.*, **182**, 1104.
Eadie, J. M. & Oxford, A. E. (1957). *Nature, Lond.*, **179**, 485.
Elsden, S. R. & Lewis, D. (1953). *Biochem. J.* **55**, 183.
Gutierrez, J. (1955). *Biochem. J.* **60**, 516.
Gutierrez, J. & Davis, R. E. (1959). *J. Protozool.* **6**, 222.
Heald, P. J. & Oxford, A. E. (1953). *Biochem. J.* **53**, 506.
Hobson, P. N. & MacPherson, M. (1952). *Biochem. J.* **52**, 671.
Hobson, P. N., Mann, S. O. & Oxford, A. E. (1958). *J. gen. Microbiol.* **19**, 462.
Hockenhull, D. J. D. & Herbert, D. (1945). *Biochem. J.* **39**, 102.
Howard, B. H. (1957). *Biochem. J.* **67**, 643.
Howard, B. H. (1959*a*). *Biochem. J.* **71**, 671.
Howard, B. H. (1959*b*). *Biochem. J.* **71**, 675.
Hungate, R. E. (1943). *Biol. Bull., Wood's Hole*, **84**, 157.
Mould, D. L. & Thomas, G. J. (1958). *Biochem. J.* **69**, 327.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Oxford, A. E. (1958). *N.Z. J. agric. Res.* **1**, 809.
Pirt, S. J. & Whelan, W. J. (1951). *J. Sci. Fd Agric.* **2**, 224.
Scarisbrick, R. (1954). *Vet. Rec.* **66**, 131.
Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.

Biochem. J. (1960) **76**, 452

Some Properties of Neutral-Salt-Soluble Collagen. 1

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Neutral-salt-soluble collagen is a protein, or group of proteins, involved in collagen anabolism (Harkness, Marko, Muir & Neuberger, 1954; Gross, 1958*a*; Jackson, 1957) and showing the unusual behaviour of aggregating to typical collagen fibrils when its solutions are warmed to body temperature (Jackson & Fessler, 1955; Gross, Highberger & Schmitt, 1955). One available method of isolation was investigated in the present study and the product characterized. The temperature-dependent ability to form fibres was in part reversible and this led to the fractionation of the material into three parts: fraction A, which formed fibres reversibly; fraction B, which did not form fibres; fraction C, which formed fibres irreversibly. The three fractions were characterized. Coupled with the investigation of the isolation procedure was an estimation of the maximum possible phosphorus content of neutral-salt-soluble collagen, and the apparent rate of incorporation of ^{32}P. This part of the work was prompted by the report of phosphohydroxylysine in calf embryo (Gordon, 1948), together with the occurrence of hydroxylysine in collagen (Van Slyke, Hiller & McFadyen, 1941; Desnuelle & Antonin, 1947), and the presence of neutral-salt-soluble collagen principally in rapidly growing animals (Gross, 1958*a*). Some of these findings have been briefly reported (Fessler, 1957), and further studies on the temperature-dependent behaviour of neutral-salt-soluble collagens are given in the next paper (Fessler, 1960).

Unless specifically stated to the contrary, the term neutral-salt-soluble collagen implies material extracted from the skins of young rabbits and purified by the methods described.

EXPERIMENTAL

Materials

Animals. (1) Steadily growing rabbits, 5–7 weeks old; (2) young rats from the laboratory stock of The Department of Biochemistry, Oxford, weighing about 80 g. and injected intraperitoneally with 50 μc of [^{32}P]phosphate/100 g. body wt.

Reagents. These were of analytical grade, except disodium ethylenediaminetetra-acetic acid, which was of best available grade. Glass-distilled water saturated with toluene was used.

Buffers. Phosphate buffer: 0·067 M-KH_2PO_4 and 0·067 M-Na_2HPO_4 or K_2HPO_4 were mixed to give buffers of pH 7·0, 7·4 and other values as required; citrate buffer was 0·067 M-citric acid–0·033 M-NaCl–0·033 M-NaOH, pH 3·7; acetate buffer was 0·067 M-acetic acid and 0·067 M-sodium acetate mixed to pH 3·6 (glass electrode); ethylenediaminetetra-acetic acid (EDTA) buffer was disodium salt, 0·62 g./l., and 0·2 M-NaCl, adjusted with 2 N-NaOH to pH 6·8 (glass electrode); this EDTA buffer was used in all experiments involving ^{32}P.

Methods

Isolation and purification of neutral-salt-soluble collagen from skin. The scheme of Jackson & Fessler (1955) was used with the following modifications. At all stages, except where mentioned, the Spinco preparative ultracentrifuge was used for clarifying solutions (usually rotor no. 20, 20 000 rev./min. for 1 hr.) and often also for collecting precipitates, particularly when EDTA buffer was used. Materials were precipitated four times by salting-out. In later preparations no sodium salts were used, only potassium compounds, so as to avoid the formation of a crystalline sodium phosphate at high salt concentrations. This substitution of cation had no effect on any of the observed properties of collagen. It was found best to add only conc. KCl solution, and not solid salt, towards the end of each salting-out process. Solutions of collagen were always kept at cold-room temperature (2–8°) during all the experiments described in this paper, unless specifically stated to the contrary. Material precipitated with salt was stored at −10° to −20°. To make up a solution from this material, a portion was gently dispersed by a 'mashing' action in a small volume of buffer of 1% acetic acid, exhaustively dialysed against buffer and cleared on the ultracentrifuge.

The preparation from guinea pig of neutral-salt-soluble collagen containing [^{14}C]glycine is described in the next paper.

Phosphorus content. This was measured by the method of Berenblum & Chain (1938). A sample (5 ml.) containing 2–4 μg. of P was ashed with 2 ml. of conc. HNO_3 and 0·5 ml. of conc. H_2SO_4 for 4–5 hr.; the residue was hydrolysed with about 5 ml. of water by heating to boiling point for 30 min.

Estimation of radioactivity. A Veall (1948) liquid counter of 10 ml. capacity was used together with standard scaling equipment. About 1000 counts were recorded for each sample, which usually took about 1 hr., and corresponding background counts were taken (9–12/min.). Samples were measured only after dialysis against three changes of buffer, and the very small radioactivity, if any, of the last change of buffer was subtracted from that measured for the sample. Radioactivities of heat gels were measured after dissolving the centrifuge precipitate in 10 ml. of the 10% acetic acid.

Preparation of plasma for estimation of radioactivity. Oxalated blood was collected at the decapitation of the animals and the cells were at once removed by centrifuging. To the supernatant was added trichloroacetic acid, to a final concentration of 6% (w/v), and the precipitated protein was filtered off.

Constituents of collagen fractions. These were kindly measured by Miss Betty Dumsha in the laboratory of Dr Jerome Gross by the methods indicated here. Hydroxyproline: Neumann & Logan (1950); proline: Troll & Lindsay (1955); hexosamine: Boas (1953); hexose: Friedmann (1949); glycine: Christensen, Riggs & Ray (1951) and Kroll (1952).

Ultracentrifuges. The following were used: (1) Spinco preparative model L, with rotors and speeds as stated in the text and operated at 2–6° unless otherwise stated. (2) Svedberg oil-turbine ultracentrifuge installed at Oxford (Philpot, 1938; Cecil & Ogston, 1948) operated at 26–28°. (3) Spinco model E ultracentrifuge operated at 4–9°. Sedimentation coefficients, $S_{20,w}$, corrected to water as solvent at 20°, were calculated by the method of Cecil & Ogston (1948). The partial specific volume of gelatin, 0·71 (Cohn & Edsall, 1943), was used in the calculation and was assumed to vary by 0·005/10°, as given by Svedberg & Pederson (1940) for an average protein. The correction of Biancheria & Kegeles (1954) for adiabatic stretching of the Spinco analytical rotor was applied.

Couette viscometer. The apparatus of Ogston & Stanier (1953) was used, modified so that the torsion head was rotated to constant position of the indicating light beam. Position of the torsion head was read to 0·1° of arc.

Double refraction of flow. The apparatus of Ogston & Stanier (1953), modified for shear rates up to 1000 sec.$^{-1}$, was used. All measurements were made in duplicate, with alternate directions of rotation.

Refractometer. The differential refractometer of Cecil & Ogston (1951) was used. Protein concentrations were calculated on the basis of an assumed specific refractive increment of 0·00184 refractive-index units/g./100 ml. (Barer, 1954), as insufficient material was available with which to determine a more reliable value.

Spectrophotometer. This was a Hilger Uvispek.

Polarimetry. A Hilger polarimeter, reading to 0·01° of arc, was used with sodium light. The temperature of the 10 cm. cell was controlled to ±0·25° by a water thermostat. The specific optical rotation is calculated as the rotation which would be caused by 10 cm. of solution containing 1 g. of material/ml.

'Heat gellation' and 'gel ratio'. To precipitate collagen as a gel by warming, samples were placed in stoppered tubes in a 37° water bath for 1 hr., unless other times or temperatures are stated. The tubes were then either gently transferred to a refrigerator (2–4° in all experiments), or the gel was quickly fragmented with a small glass rod and the precipitate sedimented in a centrifuge rotor warmed to 35°. Tubes were either Lustroid for Spinco rotor 40 (39 000 rev./min. for 20 min.), or 7·5 cm. × 1·2 cm. glass for a small-angle centrifuge (9000 rev./min. for 10 min.). Medicinal paraffin at 35° was added to fill the residual space of Spinco tubes. Supernatants were withdrawn with a Pasteur pipette. A measure of the amount of material precipitated was defined by the term: gel ratio = 100 $[(n_p - n_s)/n_p]$, where n_p is the refractive increment of the original solution with respect to the buffer against which it had been exhaustively dialysed, and n_s is the refractive increment of the supernatant against the same buffer. Where specifically stated in the text, other parameters, such as absorption at 275 mμ, were similarly used to define further 'gel ratios'.

Separation of neutral-salt-soluble collagen into fractions A, B and C. Clarified and dialysed solutions, containing 0·2–0·3 g. of collagen/100 ml. at pH 7·4 were warmed to 37° for 1 hr. in Lustroid ultracentrifuge tubes, stored in the refrigerator for 62 hr. and the residual gel (fraction C) was fragmented and centrifuged off (rotor 40, 39 000 rev./min., 20 min., 4°). The supernatant was then gelled at 37° for 1 hr., and the gel was immediately fragmented and centrifuged (rotor 40, 39 000 rev./min., 10 min., 33°): the residue was fraction A and the final supernatant fraction B. Residues A and C were gently dispersed in ice-chilled 1% acetic acid and dialysed against 1% acetic acid overnight. An appreciable part of fraction C did not dissolve. After exhaustive dialysis against phosphate buffer, pH 7·4, the solutions were clarified (Spinco rotor 40, 39 000 rev./min., 1 hr.).

RESULTS

Studies on the isolation of neutral-salt-soluble collagen

The neutral-salt-soluble collagen isolated by Jackson & Fessler (1955) was heterogeneous (Fessler, 1957), and so it became necessary to investigate the method of isolation. It is shown below that even material obtained after extended purification along the lines of Jackson & Fessler (1955) is heterogeneous.

Pooled extracts were obtained from the skins of eight young rats which, 2 hr. before being killed, had been given ^{32}P. The purification procedures of precipitation with salt were repeated on these extracts until the material attained constant properties, as judged by the following criteria: (1) the distribution of material between gel and supernatant formed on warming, in terms of (*a*) refractive increment ('gel ratio'), (*b*) absorption at 275 mμ, and (*c*) radioactivity; (2) (*a*) the total content of ^{32}P, and (*b*) the total absorption at 275 mμ; (3) the form of the u.v.-absorption curve. Total absorption at 275 mμ decreased somewhat between the last two of the four repetitions of the purification procedure. The results are shown in Figs. 1–3. It is known (Gustavson, 1956) that collagen has an affinity for phosphate ions, and although large volumes of the EDTA–sodium chloride buffer were used in the preparation, there was the possibility that the observed radioactivity was due to phosphate adsorbed on collagen. A large excess of phosphate buffer was added to parts of three samples before heat-gelling, and this should have affected the distribution of radioactivity between heat gel and supernatant, if the radioactivity was due to adsorbed phosphate. As is shown in Fig. 1, such an alteration of the distribution was obtained only in the less purified samples.

A phosphorus determination in one of the supernatants which had been formed after addition of phosphate buffer showed that only little of the added phosphate had been removed by the gel.

The practical conclusion from these experiments and evidence from Fig. 1 show that the purification method of Jackson & Fessler (1955) should be taken to three or four salt precipitations, and that further repetition is unlikely to change the material. The observed radioactivity was too low to allow detailed interpretation, but suggested that the radioactive phosphorus was not due to simple contamination with inorganic phosphate and so warranted some further study. The net zero count obtained with supernatant of sample 3 (Figs. 1, 2) is probably fortuitous, as the solution was rather dilute. The ultraviolet-absorption studies were hindered because they had to be done rapidly at room temperature, before gellation could set in, and so measurements were only taken at 2·5 mμ intervals. A number of curves similar to those shown in Fig. 3, but omitted from that Figure for clarity, were obtained on similarly prepared material. Various small peaks or shoulders were found in the range 255–270 mμ, and the minimum around 250 mμ was deeper for samples which had undergone more salt precipitations, or had been ultracentrifuged more exhaustively. The peak at 275 mμ was always definite.

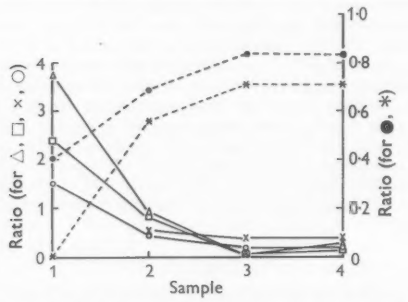

Fig. 1. Effect of successive salt precipitations of neutral-salt-soluble collagen on the distribution of material between supernatant and gel formed by warming to 37°. Abscissa represents the number of salt precipitations. Ordinates represent the following ratios: ●, (refractive increment of gel)/(refractive increment of parent solution); *, ($E_{275\,m\mu}$ of gel)/($E_{275\,m\mu}$ of parent solution); ○, (refractive increment of supernatant)/(refractive increment of gel); ×, ($E_{275\,m\mu}$ of supernatant)/($E_{275\,m\mu}$ of gel); □, (counts/hr. from supernatant)/(counts/hr. from gel); △ is a repeat of □, but excess of 0·067 M-phosphate buffer, pH 6·8, was added before warming to 37°. (Value for gel) = (corresponding value of parent solution) − (value of supernatant).

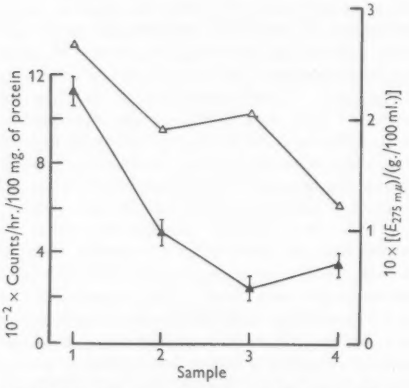

Fig. 2. Effect of successive salt precipitations on the composition of neutral-salt-soluble collagen prepared from rats injected with ^{32}P; ▲, variation of specific radioactivity with number of salt precipitations indicated as abscissa; △, corresponding specific absorption coefficients at 275 mμ.

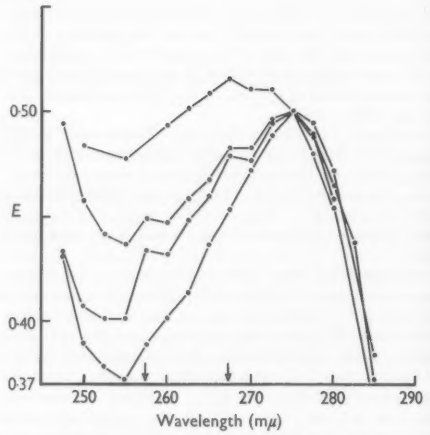

Fig. 3. Effect of successive salt precipitations of neutral-salt-soluble collagen on its absorption spectrum. All curves were corrected to $E_{275\,m\mu}$ of 0·50. Absorptions decreased in step with purification: the top spectrum was obtained after one precipitation. Arrows indicate the location of subsidiary maxima at 257 and 267 mμ.

Studies on the incorporation of radioactive phosphorus

Sets of four rats were given ^{32}P, and killed 2, 10, 18, 36 and 94 hr. after administration. Neutral-salt-soluble collagen was isolated as given in the Experimental section, with EDTA buffer and four sodium chloride precipitations. The final material

was taken up in EDTA buffer and after exhaustive dialysis clarified on the ultracentrifuge. Then it was dialysed against three changes of acetate buffer, pH 3·6, which ensured that no heat-gellation would occur during prolonged measurements at room temperature. Refractive increment, ultraviolet absorption, radioactivity and phosphorus content were measured on each sample. Radioactivity and phosphorus content were also measured on corresponding samples of the supernatant, obtained at the first sodium chloride precipitation of the skin extracts, and of samples of plasma from blood collected at death.

The results are given in Fig. 4: 100 mg. of neutral-salt-soluble collagen contained 9·2 μg. of phosphorus (mean of four samples; range 8·6–9·6 μg. of phosphorus).

Heterogeneity of neutral-salt-soluble collagen as shown by its behaviour on warming

When a solution of neutral-salt-soluble collagen is warmed, most, but not all, of the material is precipitated, i.e. the 'gel ratio' is less than 100%. From Fig. 1 it is evident that the gel ratio is not changed by purification beyond a certain point. The concentrations of materials which were gellable in different samples, obtained both from rabbit and rat, are plotted in Fig. 5 against the concentrations of the initial solutions. A straight line fitted to these points by the method of least squares indicates a common gel ratio of 84% ($83{\cdot}7 \pm 1{\cdot}5$). Slight variations of experimental procedures do not appear to alter this gel ratio substantially. Thus the pH of samples represented in Fig. 5 ranged from 6·7 to 7·4, the temperature from 28° to 37°, the method of centrifuging from ultracentrifuging at 35° to quick manipulation on a bench centrifuge at room temperature. Rabbit material prepared particularly carefully had a slightly higher gel ratio, about 88%. The material which remains in solution is not dialysable, and has been named fraction B.

The time course of the precipitation of collagen as gel at 37° is shown in Fig. 6, in which the concentration of ultracentrifuge supernatant is plotted against length of time for which the sample was incubated at 37°. Similar curves were obtained for neutral-salt-soluble collagen from rats and from guinea pigs. The standard time of warming was chosen as 1 hr., because little more material precipitated upon longer incubation (Fig. 6). The first stages of the process were followed at 22°, when the rate of precipitation is much slower, and indicated an S shape for the overall precipitation curve.

When a heat gel of neutral-salt-soluble collagen is left undisturbed in the refrigerator, the gel becomes less opaque and less rigid. This suggests that partial reversal of the heat-gellation phenomena occurs, and to investigate this the experimental

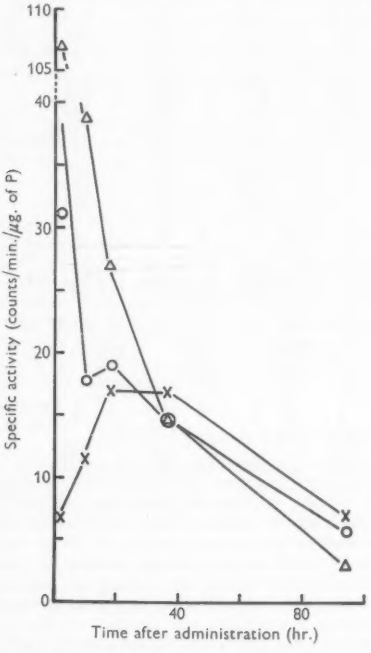

Fig. 4. Specific radioactivity of phosphorus from samples of neutral-salt-soluble collagen prepared at different times after administration of ^{32}P to rats. ×, Neutral-salt-soluble collagen; ○, residue of skin extracts after removal of neutral-salt-soluble collagen; △, plasma obtained at time of death.

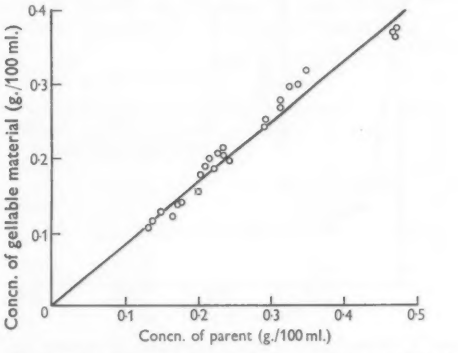

Fig. 5. Variation of the concentration of material which can be gelled with the concentration of the parent solution of neutral-salt-soluble collagen.

scheme of Fig. 7 was devised. The techniques described in the section on Methods were used. A set of 0·6 ml. samples was taken from a solution of known concentration and warmed to 37° for 1 hr. in Lustroid Spinco tubes. One tube was centrifuged at 35° and the concentration of supernatant measured; the other tubes were placed in the refrigerator. After known intervals of about 2, 4 or 6 days' cold storage, the remaining gels were centrifuged off and the refractometric concentrations of the supernatants determined. The results can be plotted as curves such as Fig. 8 (1). The total refractive increment corresponding to a point on curve (1) (Fig. 8) is composed of (*a*) refractive increment of material which did not gel on warming, (*b*) refractive increment of material which has dissolved from the gel, (*c*) any other change in refractive index of the mixture. In order to determine the concentration of redissolved material, the supernatants were warmed to 37° for 1 hr. and then centrifuged, warm, in a small-angle centrifuge. The refractive increments of the final supernatants were measured and could be plotted as curves such as Fig. 8 (2). The differences between corresponding points of curves (1) and (2) in Fig. 8 represent the concentrations of material which had gelled on warming to 37° and had subsequently dissolved on cooling: these differences are plotted as curve (3) in Fig. 8. The ordinate scale of Fig. 8 is a concentration scale, and different units of concentration may be chosen: the left scale is in g./100 ml., the right scale is in per cent, with 100% equal to the concentration of total gellable material in the original solution. If all the material which gelled originally had redissolved again, then curve (3) (Fig. 8) should rise to 100%; in fact, a plateau is reached at about 70%, consistent with the residual gel which was always found in these tubes. This suggests that the gellable material consists of two fractions: one which redissolves on cooling, named fraction A, and one which does not redissolve, named fraction C; the results of duplicate experiments are plotted in Fig. 8. Experimental difficulties are illustrated both in the scatter of points and in the rise of curve (2) with time. The errors were principally due to repeated transfers and ultracentrifuging of small volumes of solution; the refractive index would also be affected by quite small changes of water content, such as would occur on storage in Lustroid tubes and in centrifuging under medicinal paraffin. Results closely similar to those of Fig. 8 were obtained with guinea-pig neutral-salt-soluble collagen containing

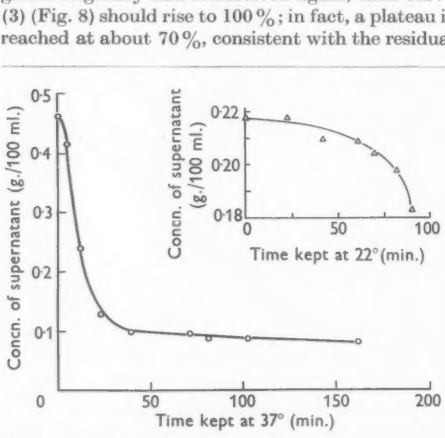

Fig. 6. Time course of the precipitation of neutral-salt-soluble collagen in 0·067 M-phosphate buffer. The large curve shows results obtained at pH 7·4 and 37°. The insert shows the initial stages of precipitation at pH 6·8 and 22°.

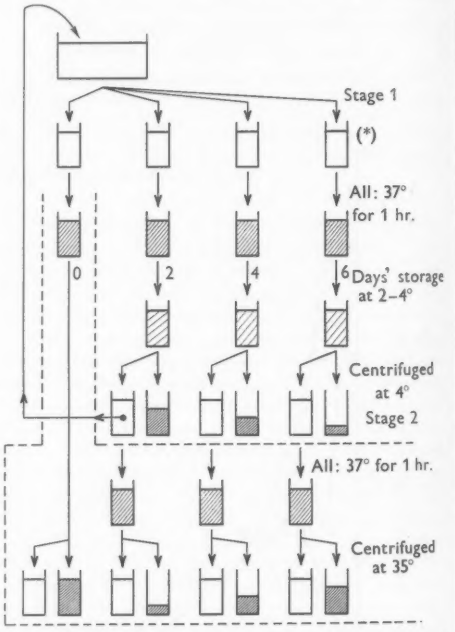

Fig. 7. Scheme of alternate warming and cooling of neutral-salt-soluble collagen. Stage 1 represents division of initial solution into several samples of 0·6 ml. and one sample (*) which contains enough solution for recycling experiments. Gels formed on warming are indicated as shaded areas. Density of shading represents the opacity of a gel, which decreases on cold storage. At stage 2 each sample is divided into a supernatant solution and a sedimented gel of collagen, the size of each shaded area representing the relative amount of gel. Most of the supernatant corresponding to sample (*) of stage 1 is made the initial solution for a further experimental cycle. Solutions are analysed for gellable collagen by the scheme enclosed with broken lines.

[^{14}C]glycine and by using radioactivity in place of refractive index as a measure of concentration.

This method of analysis by change of phase with temperature was repeated, varying the sample, the initial concentration of material and the pH of the phosphate buffer. The results are collected in Table 1. The concentration of fractions A and C were determined on three or four plateau points, equivalent to curve (3) (Fig. 8), and the values after storage for about 100 hr. in the refrigerator are given; after 150 hr. there was sometimes a further slight rise in the curve corresponding to some more gel dissolving.

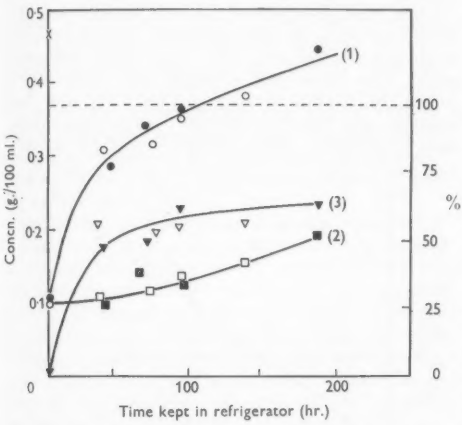

Fig. 8. Time course of the re-solution of collagen fibres at 2–4°. Solid and open symbols are results from duplicate experiments. Solutions of neutral-salt-soluble collagen, having concentration ×, were warmed to 37° for 1 hr. and then kept at 2–4°. Curve (1) shows the increase in the concentration of total material in solution with time. The concentration of material in solution was decreased to the values of curve (2) on warming to 37° for 1 hr. corresponding isolated solutions represented on curve (1). Curve (3) is a plot of the difference between corresponding values of curves (1) and (2). Left-hand ordinate is the refractometric concentration measured. Right-hand ordinate is on a % scale, with 100% as the concentration of all the material which precipitated on initially warming to 37° for 1 hr.

The behaviour of fraction A in the absence of fraction C was studied by recycling solutions through the scheme of Fig. 7. As before, samples of the solution were heat-gelled and allowed to redissolve by standing in the refrigerator. A sample (8 ml.) was used for the portion that was removed after 2 days from the refrigerator and centrifuged in the cold. The supernatant formed the starting material for a further complete analysis by the scheme of Fig. 7, and the whole process was repeated once more in one experiment. The results are given in Fig. 9 and are plotted as a percentage of gellable material redissolved against time, i.e. analogous to curve (3) of Fig. 8; for each curve the 100% unit of concentration is the total gellable material in the starting solution of that cycle. The gels formed in the second and third cycles of the scheme of Fig. 7 seemed to disappear completely on standing in the refrigerator, and the curves of Fig. 9 are in agreement with this. Recovery of gellable material must in part have been affected by losses during the repeated handling. The total evidence for the existence of the three separate fractions A, B and C will be evaluated in the Discussion section.

Solution of neutral-salt-soluble collagen in acetic acid and acetate buffer

Gels of neutral-salt-soluble collagen can be dissolved in dilute acetic acid and this was made use of in separating the fractions of neutral-salt-soluble collagen. The following experiments showed that re-solution via dilute acetic acid did not alter (1) the amount of material gelled at 37° (at pH 7), (2) the specific optical rotation and (3) that true solution occurred as judged by ultracentrifuging.

Table 1. *Composition of different samples of neutral-salt-soluble collagen*

Samples were dialysed against 0·067 M-phosphate buffer of stated pH and their concentrations were adjusted to the tabulated initial values. Compositions were then determined by the method given in the text. For preparation of fractions A, B and C see text.

		Concentration (g./100 ml.)				Percentage composition		
			Fraction			Fraction		
Sample	pH	Initial	A	B	C	A	B	C
I	7·4	0·463	0·233	0·093	0·137	50	20	30
II	7·2	0·327	0·154	0·033	0·140	47	10	43
III	7·0	0·346	0·177	0·029	0·139	51	9	40
IV	7·0	0·238	0·110	0·044	0·084	46	18	35
V	7·0	0·192	0·093	0·047	0·058	49	24	30
VI	7·0	0·163	0·071	0·040	0·053	43	24	32
VII	6·8	0·314	0·180	0·048	0·086	57	15	27
VIII	6·65	0·334	0·183	0·034	0·116	55	10	35
IX	6·5	0·314	0·161	0·037	0·117	51	12	37

(1) A portion (0·72 ml.) of a known solution of neutral-salt-soluble collagen in phosphate buffer, pH 7·0, was placed in a dialysis sac, and this in a small volume of buffer in a test tube. The whole was warmed to 37° for 75 min. and then cooled to 4°, care being taken throughout not to disturb the sac. This was now gently transferred to cold 1% acetic acid and after dialysis for 2 hr. the contents were fluid and clear. After exhaustive dialysis against phosphate buffer, the contents of the sac were quantitatively collected and weighed. The clear solution was ultracentrifuged (rotor 40, 39 000 rev./min., 40 min.) and its gel ratio determined in the usual way, as was that of a control. The calculated total gellable material placed into the sac was 1·40 mg. and that recovered was 1·39 mg.

(2) The previous experiment was repeated with a larger volume of solution; the initial and final solutions were dialysed to pH 7·4 and their specific optical rotations measured: start −376°, end −378°.

(3) In the cold a solution of neutral-salt-soluble collagen was exhaustively dialysed against acetate buffer, pH 3·7. In the analytical ultracentrifuge a sample showed only the usual single collagen peak. The remaining solution was centrifuged (rotor 40, 39 000 rev./min., 1 hr., 4°) and no material was thereby removed: initial concentration 2·20 mg./ml., final concentration 2·19 mg./ml.

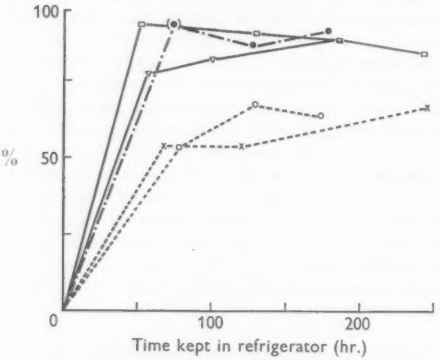

Fig. 9. Percentage re-solution at 2–4° of fibres of neutral-salt-soluble collagen cycled repeatedly through the scheme of Fig. 7. First cycle: ○, ×; second cycle: □, ▽; third cycle: ●. The value of the point shown in parentheses is uncertain.

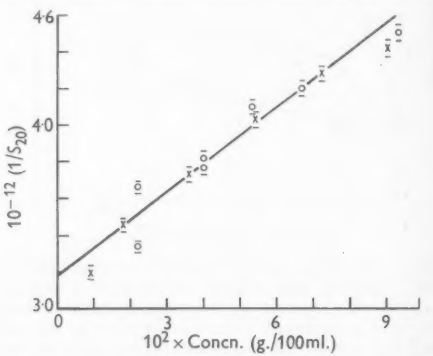

Fig. 10. Variation of reciprocal of sedimentation coefficient of neutral-salt-soluble collagen with concentration at 4–9° (○) and 26–28° (×). Bars indicate the standard deviations of values (preparation P.1).

Table 2. *Summary of physical measurements on neutral-salt-soluble collagen*

Native materials were separate preparations which had not been fractionated. 'Re-dissolved' material had been subjected to the fractionation procedure without separating the fractions. Concentrations are in g./100 ml. $[\eta]_c$ = intrinsic viscosity = $\underset{c\to 0}{\mathrm{Lt}}\left(\frac{[\eta_0-1]}{c}\right)$, where $\eta_0 = \underset{G\to 0}{\mathrm{Lt}}\,(\eta_R)_G$, $(\eta_R)_G$ = relative viscosity at shear rate G; $J = a/b$, where a = semimajor and b = semiminor axis of prolate ellipsoidal particle of effective specific volume V', particle weight M and rotary diffusion coefficient θ. For preparation of fractions A, B and C see text.

Preparation	$10^{13}\,S^{\circ}_{20,w}$	$10^{-13}\left[\frac{d\,(1/S_{20,w})}{dc}\right]$	$[\eta]_c$	θ (sec.$^{-1}$)	J	V' (ml./g.)	$10^{-5}M$	$2a$ (Å)	$2b$ (Å)
Native									
P.1	3·23	1·68	47	220	42	36	9·8	3400	80
P.2	2·76	1·03	28	—	43	19	5·5	—	—
P.3	2·76	0·81	57	360	83	14	6·2	3100	40
Fraction A									
P.3.A	2·87	1·08	20	440	35	21	4·4	2600	75
Fraction B									
P.3.B	2·97	0·33	60	—	152	5	5·9	—	—
Fraction C									
P.3.C	2·84	0·99	33	—	50	19	5·1	—	—
'Re-dissolved'									
P.3.K	2·86	0·94	36	—	55	18	5·1	—	—

Physical properties of neutral-salt-soluble collagen and of separated fractions A, B and C

Sedimentation, Couette viscosity and streaming birefringence measurements were made on three separate preparations (P.1, P.2, P.3), of neutral-salt-soluble collagen, on the fractions A, B, C into which one of these (P.3) was divided, and on a control sample (P.3.K) in which P.3 was subjected to a process used in fractionation, namely gelling at 37° for 1 hr. and subsequent re-solution via 1% acetic acid. Preparation P.1 had not been clarified by ultracentrifuging. Samples were kept at 4° throughout, except during sedimentation in the Svedberg ultracentrifuge and during refractometry at room temperature. Each preparation was usually investigated at five concentrations (0·01–0·09 g./100 ml.) and the following properties were always found.

Sedimentation. Schlieren diagrams consisted of a single sharpened peak. Sedimentation behaviour was the same in the cold (4–9°, Spinco) and at room temperature (26–28°, Svedberg) when compared for one preparation, P.1, as is shown, after the usual correction to water at 20°, in Fig. 10. Sedimentation coefficients at zero concentration $S^{\circ}_{20,w}$ were obtained by extrapolation of similar linear plots of $1/S_{20,w}$ against concentration and are given in Table 2.

Viscosity. Relative viscosity $(\eta_R)_G$ varied with shear rate G, an example being given in Fig. 11. At greater dilutions $(\eta_R)_G$ was independent of shear rate at all but the lowest shear rates. Relative viscosities at zero shear rates η_0 were obtained by extrapolation of $1/(\eta_R)_G$ against G. The specific viscosities $[\eta_0 - 1]/c$ were found to be independent of concentration, at concentrations less than 0·08 g./100 ml. The intrinsic viscosities

$$[\eta]_c = \left\{ \operatorname{Lt}_{c \to 0} \frac{[\eta_0 - 1]}{c} \right\}$$

are given in Table 2.

Flow birefringence. At a given shear rate, G, the angle of isocline, χ, varied with concentration of the sample, and was linearly extrapolated to zero concentration. The rotary diffusion coefficient θ was calculated for a long rod from χ and G by the treatment of Scheraga, Edsall & Gadd (1951). The variation of θ with G is shown for some samples in Fig. 12.

Calculation of molecular parameters. Particle weight, hydration, axial ratio and length were calculated by the 'porous-plug' treatment of Ogston (1953) and are given in Table 2. With the axial ratio obtained in this way, the length of the particles was also calculated from flow-birefringence measurements by the method of Scheraga *et al.* (1951). For these calculations a partial specific volume of 0·71 and a specific refractive

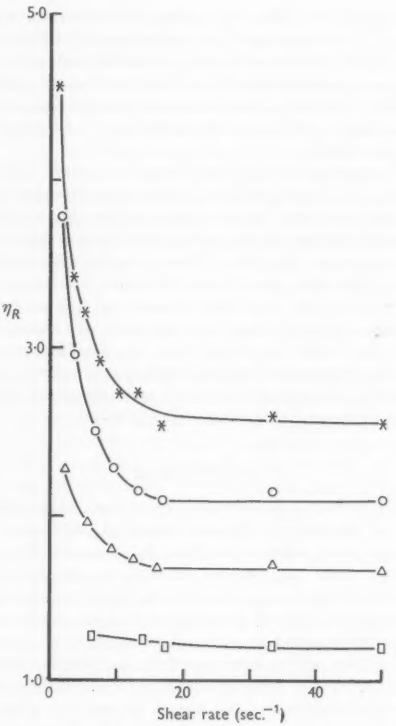

Fig. 11. Variation of relative viscosity (η_R) of solutions of neutral-salt-soluble collagen with shear rate at different concentrations (g./100 ml.): *, 0·08; ○, 0·06; △, 0·04; □, 0·015.

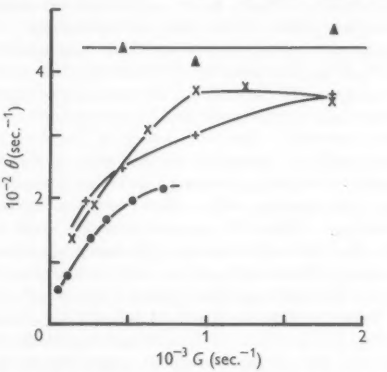

Fig. 12. Variation of rotary diffusion coefficient θ with shear rate G of neutral-salt-soluble collagen: sample P.1 (●), sample P.3 (×) and the fractions A (▲) and C (+) into which sample P.3 was separated.

increment of $1{\cdot}84 \times 10^{-3}$ refractive-index units/g./100 ml. were assumed, as experimental difficulties precluded obtaining more accurate values. The assumed partial specific volume is that of gelatin (Cohn & Edsall, 1943), and the specific-refractive increment value is representative of proteins (Barer, 1954).

Composition of separated fractions A, B and C. Proline, hydroxyproline, glycine, hexosamine and hexose contents were measured by Miss Betty Dumsha in the laboratory of Dr Jerome Gross on the separated fractions of two samples. No significant differences were found between the fractions, no hexosamine and only traces of hexose were found. Hydrolysates of fraction B contained material which interfered with the glycine estimation by the method of Christensen *et al.* (1951), and therefore glycine was isolated and estimated as the dinitrobenzene derivative (Kroll, 1952).

DISCUSSION

The author's principal interest was in the ability of an inherently fibrous material, collagen, to change from solution to fibre by warming from 4° to 37°, and the partial, though incomplete, reversal of this change on cooling. The initial investigations (Jackson & Fessler, 1955) were made with material isolated by the salt-precipitation method of Jackson and it was therefore necessary to study this method in greater detail. The possibility that phosphohydroxylysine, found in calf by Gordon (1948), could be a constituent of such material was investigated at the same time.

Isolation of neutral-salt-soluble collagen and its composition. From the results given in Figs. 1 and 2 it is concluded that repeated salting-out of neutral-salt-soluble collagen gives a product of constant properties after three such precipitations. Contamination with nucleic acid-like material (u.v. curve, Fig. 3) is thereby successively decreased to the limits of detection, hexosamine-containing materials are eliminated and hexose is decreased to trace amounts. Jackson, Leach & Jacobs (1958) have made a complete amino acid analysis on material similarly isolated, and the composition was comparable with other isolated collagens (Kendrew, 1954). The present analyses were made only to detect any relative differences between the separated fractions, and no such differences were found. The high specific optical rotation of $-377°$ is similar to that of other isolated soluble collagens (Cohn, 1955). How far the isolation procedure has affected the original state of aggregation of the material *in vivo* is not known, though the possible disruptive effects of high ionic strength and acid pH, used in other extractions, were avoided. The salted-out precipitates were amorphous under the electron microscope. Some irreversible aggregation could have been caused during salting out and storage, as subsequent resolution was at times incomplete.

Phosphorus content and rate of incorporation of ^{32}P. A small but definite phosphorus content of material isolated by salt precipitation is indicated from the results (Figs. 1 and 2) and was determined. This phosphorus could be due to (1) adsorbed inorganic phosphate, (2) phosphorus constituent of some trace contaminant, in particular a nucleic acid, (3) phosphorus as a true constituent of these collagens. Swamping experiments with 'cold' phosphate buffer make simple adsorption unlikely, though it cannot be excluded. Contamination with nucleic acid is a very possible explanation, the measured phosphorus content being within the error of the final u.v.-absorption curves, which indicated absence of nucleic acids. When, however, the material was gelled at 37°, the radioactivity was distributed between solution and solid in the same proportion as the bulk collagenous material (measured by refractive increment). The relationship between time after administration of radioactive phosphate and the specific activity of the phosphorus in the corresponding sample of purified collagen (Fig. 4) was similar to that obtained by Harkness *et al.* (1954) for the incorporation of [^{14}C]glycine into such collagen. Lastly, the curves of Fig. 4 show that simple, random contamination of the extracted collagen did not occur, as the specific activity of the skin residues was at times lower than that of collagen, though contamination by a small amount of highly active material was certainly not ruled out. If phosphorus were a true constituent of neutral-salt-soluble collagen, there would, according to the results, be one atom of phosphorus per particle of 340 000 molecular weight units. Such a particle would contain seven hydroxylysine residues (Kendrew, 1954), but only one of these could be phosphorylated. Phosphohydroxylysine is thus not likely to be a constituent, or a constituent of any importance, of collagen.

Heterogeneity of neutral-salt-soluble collagen as shown by its behaviour on warming and cooling. The results given in Figs. 5–9 and Table 2 lead to the conclusion that in solutions of neutral-salt-soluble collagen three fractions could be distinguished: fraction A, which formed fibres at 37°, that completely dissolved again on cooling; fraction B, which did not precipitate at 37°; fraction C, which irreversibly formed fibres at 37°. (If fibres of fraction C do redissolve at 4°, they do so extremely slowly.) Complex solubility phenomena are likely in a system of rod-like particles that aggregate in an integrated manner into long fibres, and so the concept of separate fractions should be viewed with scepticism. Within the range in which the material

could be studied, however, the relationship between the fractions was independent of absolute concentration, pH and time, provided that sufficient time for equilibration had been allowed (e.g. Figs. 5 and 6 and Table 1); constancy of relationship was indicated when concentrations were expressed in relative percentage form. Moreover, if the sequence of warming and cooling was repeated according to the scheme of Fig. 7. reproducible results were obtained, as shown in Fig. 9; that is, once the initial portion of fraction C (irreversible fibre formation) was removed, all subsequently made fibres dissolved on cooling and the proportion of material which did not form fibres (fraction B) also remained constant. Further, such quantitative behaviour was shown independently of the volumes of solution used, nature of containing vessels, gross appearance of the gels and, within limits, of temperature and time of warming. Thus it is unlikely that the observed fractions were simply artifacts inherent in the polymerization process which occurred at 37°. Certainly the first warming to 37° could have wrought some irreversible change. The matter will be further discussed in a following paper (Fessler, 1960), in which further evidence is considered. The principal conclusions are that (1) on the basis of warming and cooling the material appears to be heterogeneous, and (2) that a part of neutral-salt-soluble collagen reversibly forms fibres, according to temperature (previously reported, Fessler, 1957). Gross (1958*b*) extended the time of incubation at 37° to as much as 24 hr. and found that on subsequent cooling the re-solution of his collagen was greatly decreased in neutral buffers. He made the suggestion that upon prolonged maintenance of collagen at 37° the constituent particles of fibres become arrayed in a progessively more ordered crystalline state, making subsequent re-solution more difficult. Although this may possibly be so, the present findings cannot be fully interpreted along these lines: successive incubations at 37° for 1 hr. of a given sample should yield equal proportions of material which does not dissolve on cooling, whereas this occurred only on the first incubation (fraction C, see scheme of Figs. 7 and 9). Both rate and equilibrium processes are involved: to obtain full re-solution of the fibres of fraction A the material had to be kept for at least 48 hr. at 4° (Fessler, 1957) whereas Gross (1958*c*) principally used overnight storage in the cold.

Physical properties of neutral-salt-soluble collagen and re-solution of precipitates in dilute acetic acid. The variations of rotary diffusion coefficient (θ) with shear rate (G), shown in Fig. 12, indicate heterogeneity of all the materials studied, with the possible exception of fraction A. The single peak of the ultracentrifuge schlieren diagrams, together with the evidence discussed above, lead one to regard this heterogeneity as due to a range of particles of basically one material, rather than due to radically different materials. Boedtker & Doty (1956) found with acid-soluble fish collagens that homogeneous material occurred only in the supernatant of an extensively ultracentrifuged solution, all other material being discarded. Such extensive ultracentrifuging would have narrowed the sampling more than was desired in the present study. The particle parameters given in Table 2 therefore represent some form of average and hence little was to be gained by any more refined treatment of calculation; similarly, the assumptions made in choosing partial specific volume and specific refractive increment cannot have introduced any appreciable error.

The conclusions drawn from the results given in Table 2 are that the neutral-salt-soluble collagens are highly elongated particles, of axial ratios of the order of 50, a few thousand Ångström units long and with a particle weight of some 500 000. Given the estimated length, the magnitude of the hydrodynamically hydrated volume suggests a rod-like particle. Contamination of any one sample with a relatively small amount of a higher aggregate of such nature would have increased the relative viscosities measured at low shear rates and decreased the apparent rotary diffusion coefficients, and as a result the stated particle weight, length and axial ratio would be somewhat higher than the representative values. Considerable particle–particle interaction was indicated both by the shape of the sharpened schlieren peak in the sedimentation diagram and by the variation of the angle of isocline at a given shear rate with concentration. The effects of such interactions should have been minimized by the extrapolation of all values to zero concentration. Differences in the parameters calculated for the different samples are on the whole not likely to be significant. The largest particle weight of $9{\cdot}8 \times 10^5$ was found for the crudest preparation P.1, which had been obtained before a preparative ultracentrifuge was available.

The difference between P.3 and P.3.K, which was made from P.3 by gellation (without fractionation) and re-solution in 1% acetic acid, suggests that dilute acetic acid may have some dispersing action on possible aggregates. Under the specified conditions, treatment with dilute acetic acid did not cause degradation to gelatin, as both the amount of gellable material and the specific optical rotation were not thereby changed. Although ultracentrifuge studies showed that true solution of these neutral-salt-soluble collagens occurred in 0·067 M-acetate buffer, pH 3·6, this was found not to occur in 0·067 M-citrate buffer of similar pH, which gave a dispersed material that was in part readily

removed by ultracentrifuging. This stands in contrast with the acid-soluble collagens that have been studied in such citrate buffers. It may be significant that not all of the heat gel of fraction C could ever be dissolved in 1% acetic acid, and further that some of the material which did dissolve was subsequently precipitated on exhaustive dialysis against phosphate buffer. The results on measurements of fraction C can thus only be representative of a part of it. In contrast, all of fraction A was recovered by such re-solution procedure. The particle weight and length calculated for fraction B may easily be too large, as only dilute solutions of this fraction were available and little contamination with higher aggregates would have appreciably affected the results.

No striking differences between the three fractions were thus observed by the physical methods employed.

Further discussion is in the corresponding section of the following paper. It must be emphasized that the results and conclusions of this work may only be applicable to the particular materials used, as unfortunately we know little about the relationships between the various collagens which different investigators have studied in solution. The generic name neutral-salt-soluble collagen does not imply identity; indeed the materials used here have different solubility properties from those isolated by Gross *et al.* (1955) from calf skin.

SUMMARY

1. Neutral-salt-soluble collagen was extracted from the skins of young rabbits and rats and purified by repeated salting-out. The purification process was studied.

2. This neutral-salt-soluble collagen was separated into three parts: (*a*) fraction A, which reversibly formed fibres at 37°, the fibres dissolving again on cooling, (*b*) fraction B, which did not form fibres on warming to 37°, and (*c*) fraction C, which on warming to 37° formed fibres that did not dissolve on subsequent cooling.

3. The proportions of these fractions were found to be independent of concentration and pH over a limited range.

4. Evidence for the existence of three fractions was obtained from a study of the effects of repeated warming and cooling.

5. The original material and the separated fractions were investigated by sedimentation, viscosity and streaming birefringence analyses. No significant differences were found between the fractions. A representative particle was estimated as a highly elongated rod, several thousand Ångström units long, with a particle weight of about 500 000.

6. No differences in the hydroxyproline, proline or glycine contents of the separate fractions were found. Hexose and hexosamine contents were negligible.

7. Neutral-salt-soluble collagen was found to contain not more than one atom of phosphorus in a particle of 340 000 molecular-weight units. The phosphorus is probably present as an impurity.

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REFERENCES

Barer, R. (1954). *Quart. J. micr. Sci.* **95**, 339.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Biancheria, A. & Kegeles, G. (1954). *J. Amer. chem. Soc.* **76**, 3737.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Boedtker, H. & Doty, P. (1956). *J. Amer. chem. Soc.* **78**, 4267.
Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
Christensen, H. N., Riggs, T. R. & Ray, N. E. (1951). *Analyt. Chem.* **23**, 1521.
Cohn, C. (1955). *J. biophys. biochem. Cytol.* **1**, 203.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p. 374. New York: Reinhold Publishing Corp.
Desnuelle, P. & Antonin, S. (1947). *Biochim. biophys. Acta*, **1**, 50.
Fessler, J. H. (1957). *Fed. Proc.* **16**, 37.
Fessler, J. H. (1960). *Biochem. J.* **76**, 463.
Friedmann, R. (1949). *Biochem. J.* **44**, 117.
Gordon, A. H. (1948). *Nature, Lond.*, **162**, 778.
Gross, J. (1958*a*). *J. exp. Med.* **107**, 265.
Gross, J. (1958*b*). *Nature, Lond.*, **181**, 556.
Gross, J. (1958*c*). *J. exp. Med.* **108**, 215.
Gross, J., Highberger, H. J. & Schmitt, F. O. (1955). *Proc. nat. Acad. Sci., Wash.*, **45**, 1.
Gustavson, K. H. (1956). *The Chemistry and Reactivity of Collagen*, chapt. 4, 5. New York: Academic Press Inc.
Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.
Jackson, D. S. (1957). *Biochem. J.* **65**, 277.
Jackson, D. S. & Fessler, J. H. (1955). *Nature, Lond.*, **176**, 69.
Jackson, D. S., Leach, A. A. & Jacobs, S. (1958). *Biochim. biophys. Acta*, **27**, 418.
Kendrew, J. C. (1954). In *The Proteins*, vol. 2B, p. 920. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Kroll, S. (1952). *Biochem. J.* **52**, 227.
Neumann, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **215**, 655.

Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.
Philpot, J. St. L. (1938). *Nature, Lond.*, **141**, 283.
Scheraga, H. A., Edsall, J. T. & Gadd, J. O. (1951). *J. chem. Phys.* **19**, 1101.
Svedberg, T. & Pederson, K. O. (1940). *The Ultracentrifuge*, Appendix II. Oxford: University Press.
Troll, W. & Lindsay, J. (1955). *J. biol. Chem.* **215**, 655.
Van Slyke, D. D., Hiller, A. & MacFadyen, D. A. (1941). *J. biol. Chem.* **141**, 681.
Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.

Biochem. J. (1960) **76**, 463

Some Properties of Neutral-Salt-Soluble Collagen. 2

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It was shown in the preceding paper (Fessler, 1960) that a purified preparation of neutral-salt-soluble collagen could be separated into three closely similar fractions. The significant difference between these fractions was their solubility behaviour at 4° and 37°: fraction B remained in solution at both temperatures, fractions A and C were in solution at 4° and formed typical collagen fibres at 37°, the fibres of fraction A completely dissolved again on subsequent cooling but those of fraction C did not. The physical and possible physiological relationships between these fractions have been further explored and the nature of the temperature-dependent process of fibre formation studied.

EXPERIMENTAL

Unless specifically stated, the experimental techniques were those previously described (Fessler, 1960).

Preparation from guinea pig of neutral-salt-soluble collagen labelled with [^{14}C]*glycine.* Suckling animals, which had steadily been gaining weight to a final weight of 160–225 g., were each given 100 μc of [^{14}C]glycine intraperitoneally and killed 6–7 hr. later by heart puncture. The hair was clipped off and skins, free of muscle layers, were removed and minced in a chilled grinder. The mince was shaken overnight with twice its weight of buffer (0·12 M-KCl–0·016 M-K_2HPO_4,–0·004 M-KH_2PO_4, pH 7·4) and the suspension was centrifuged (Spinco rotor 21, 15 000 rev./min., 2 hr.). The supernatant was kept and the skin residues were re-extracted by the same process. Neutral-salt-soluble collagen was isolated from the pooled supernatants by repeated salting-out as previously described (Fessler, 1960).

Contamination of the purified neutral-salt-soluble collagen by [^{14}C]glycine was ruled out by the following experiment. To a solution of 0·6 mg. of neutral-salt-soluble collagen, equivalent to $1{\cdot}6 \times 10^4$ counts/min., was added 10 mg. of glycine and the mixture was dialysed against phosphate buffer. The maximum total radioactivity in the external medium was 70 counts/min., and more than 80% of the glycine expected at equilibrium was recovered from the external medium [the glycine was isolated by chromatography on Dowex 50 and estimated by the method of Yemm & Cocking (1955)].

Neutral-salt-soluble collagen from rabbit. This was prepared as described by Fessler (1960).

Acid-soluble collagen from rabbit. The skin residues left after five extractions of neutral-salt-soluble collagen with 0·067 M-phosphate buffer, pH 7·0 (Fessler, 1960), were extracted with 0·067 M-citrate buffer, pH 3·7. Collagen was precipitated from the clarified extract by adding NaCl to 5% (w/v). The precipitate was redissolved in citrate buffer and precipitated by further addition of NaCl. This was repeated twice. Finally, the material was precipitated by dialysis of the citrate solution against tap water, followed by distilled water.

Radioactivity of protein. This was measured as the time required for 1000 or 10 000 counts to be recorded by a gas-flow counter on 0·1 or 0·2 ml. of sample evenly dried as an 'infinitely thin' layer on a planchet. The linear relationship between counts/min. and concentration, obtained on a set of five serially diluted samples, had a standard deviation of 2·5%.

Specific radioactivity of the glycine of collagen. Equal parts of collagen solution and conc. HCl were mixed and, after hydrolysis at 135° for 3 hr., water and HCl were removed *in vacuo*. The amino acids in duplicate samples of hydrolysate, corresponding to 300 μg. of collagen each, were converted into 2:4-dinitrophenyl (DNP) derivatives and DNP-glycine was isolated by the method of Kroll (1952); the final solution was made in N-NH_3 solution in place of the bicarbonate solution used by Kroll. Absorption at 365 mμ and radioactivity were measured on a set of several dilutions of each ammoniacal solution. For plating out, carageenin (Seaplant Chemical Corp., New Bedford, Mass., U.S.A.) was added to 0·01% final concentration and 0·9 ml. of the mixture was evaporated on planchets in duplicate.

Solubility measurements on fraction A. Fraction A was isolated from ^{14}C-labelled neutral-salt-soluble collagen of

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guinea pig by the method of Fessler (1960), exhaustively dialysed against 0·067 M-phosphate buffer, pH 6·8, and the concentration was determined refractometrically (500 μg./ml.). Samples (0·5 ml. or less) were placed in Lustroid tubes (approx. $1\frac{1}{2}$ in. × $\frac{3}{8}$ in. for Spinco rotor no. 40 with adaptor), well stoppered and incubated in a water bath at 27–37° as required, together with liquid paraffin and the Spinco rotor. At the end of incubation tubes were topped up with liquid paraffin, sealed and at once centrifuged (30 000 rev./min. for 10 min.). Most of the liquid paraffin was then removed, and the stoppered tubes were kept in the water bath. Samples (0·1 ml.) were rapidly plated out, the pipette was washed three times with 10% (v/v) acetic acid and the washings were added to the planchets.

Concentration of solutions of fraction B. A dilute solution of fraction B in 0·067 M-phosphate buffer, pH 7·4, was isolated as previously described (Fessler, 1960). About 85% of the water was removed under controlled conditions *in vacuo*, the temperature of the solution being maintained at 7–11° throughout. A fibrous precipitate formed. The suspension was quantitatively transferred to a dialysis bag and practically all the material dissolved at 2–4° after exhaustive dialysis against 0·067 M-phosphate buffer, pH 7·4. The solution was cleared on the ultracentrifuge (30 000 rev./min. for 90 min.).

Determination of 'precipitation time' of neutral-salt-soluble collagen. Solutions of neutral-salt-soluble collagen and of various substances were added to a set of small test tubes (approx. 5 cm. × 0·5 cm.) chilled in ice. The contents of each tube (0·5 ml. or less) were stirred with a small glass rod. Tubes, together with their stirrers, were quickly placed in a water bath with a glass front. The tubes were stirred in turn. In due course a faint haze would appear in each tube, which could usually be quickly coalesced to a precipitate and then to a clot adhering to the small rod. The time between immersion of a tube and the appearance of a definite precipitate was noted and called 'precipitation time'. Because of a system of randomization, the observer did not know what a tube contained while he determined its 'precipitation time'.

Preparation of material for electron microscopy. A drop of the solution or suspension to be investigated was placed on a specimen grid covered with nitrocellulose film. For a few minutes the grids were supported on a microscope slide above a little water in a closed Petri dish. All materials could be kept chilled in ice, or at 37°. Liquid was blotted off with filter paper and replaced with 1% phosphotungstic acid, and finally the grids were washed twice in water.

RESULTS

Experiments to elucidate the nature of fraction B

Fraction B contains all the material which does not precipitate on warming a solution of neutral-salt-soluble collagen to 37° for about 1 hr., and both chemical and physical analyses had shown it to be similar to the fractions which precipitate as fibres under these conditions (Fessler, 1960). Experiments were made to see whether fraction B could be one or more of the following: (i) a suspension of fine fibrils of fractions A and C; (ii) a saturated solution of collagen in equilibrium with the fibres of fractions A and C; (iii) a solution of gelatin.

Ultracentrifuging was used in the separation of the fractions and this made contamination of fraction B with fibres unlikely. No fibres were detected in a number of samples of fraction B examined in the electron microscope after removal of the other fractions, provided that solutions placed on the electron-microscope grids were protected from evaporation. Some fibres were found if samples were slowly evaporated on to specimen grids by incubating for several hours in a moist atmosphere at 37°. Large quantities of typical striated collagen fibres were found in the precipitates formed from fraction B upon slow concentration, at 7–11°, of its dilute solutions (see Experimental section). Thus although solutions of fraction B were not initially contaminated with fibres, fibres were formed when the ionic strength of the buffer solutions was increased by evaporation.

In the preparation of fibres from solutions of fraction B, the determining factor was the salt concentration and not the concentration of collagen. Thus most of the fibrous precipitate of fraction B, formed on concentrating its solution at 7–11°, dissolved again after dialysis against the usual 0·067 M-phosphate buffer at pH 7·4. The concentration of such enriched solutions of fraction B was about 700 μg./ml. At this concentration solutions of fraction A form a gel on warming to 37° for 1 hr., and definite precipitates are still formed by solutions of fraction A containing 100 μg./ml. or less. In contrast, no precipitate appeared on warming solutions of fraction B (770 μg./ml.) to 37° for 1–24 hr. (Prolonged incubation does not visibly alter the corresponding gels of fraction A.) This finding was quantitatively confirmed. Samples (2 ml.) of fraction B (650 and 765 μg./ml.) were kept at 37° for 1 hr., ultracentrifuged at 35° (Spinco rotor no. 40, 20 000 rev./min. for 25 min.) and samples of the supernatants were carefully removed and their concentrations were determined refractometrically (700 and 770 μg./ml. respectively). No decrease in the amount of material in solution had occurred, whereas in corresponding experiments with fraction A 93% of the total material was removed.

One possible method of distinguishing between the soluble collagens and gelatin is the effect of low ionic strength on their solubilities: gelatin is soluble in water, the collagens are not. On dialysis against water, fraction B was found to be quantitatively precipitated. Fraction B (3 ml.) containing 1·93 mg. of collagen when exhaustively dialysed against water formed a voluminous precipitate. This was quantitatively separated on the ultracentrifuge, dissolved in 1% acetic acid and estimated refractometrically after dialysis against

phosphate buffer; 1·93 mg. of collagen was recovered from the precipitate and the supernatant contained less than 0·01 mg. of material.

Interaction of fractions A and B would be possible. To test this, about 0·4 g. of a solution of fraction A (6650 μg./ml.) and 1·4 g. of a solution of enriched fraction B (735 μg./ml.) were accurately weighed out in duplicate, mixed and the final refractive increment was checked. After warming to 37° for 1 hr., the precipitate was removed in the usual way at 36° on the ultracentrifuge and the refractometric concentration of the supernatant determined. Table 1 shows the results together with corresponding values calculated for the condition that: (i) 100% of the added fraction A was precipitated; (ii) 92% of the added fraction A was precipitated; (iii) 92% or 83% of the total collagen was precipitated; (iv) the proportion of fraction B which would have to behave as fraction A, to account for the results on the basis of 92% precipitation of the total of A present, is given.

Solubility measurements on fraction A

The solubility of isolated fraction A was studied in order to determine how large a part of fraction B might represent a solution of fraction A. The effects of temperature and concentration were investigated. Equilibrium conditions were approached from opposite sides, wherever possible, by making use of the change of solubility of neutral-salt-soluble collagen with temperature. For example, in a preliminary study, one set of samples was incubated at 27°. Flocculent precipitates formed and after 20 hr. the concentration of material in solution had decreased from 500 to 160 μg./ml. Concurrently, a duplicate set was first incubated at 37° for 75 min., when firm, opaque gels formed and the concentration of material in solution decreased from 500 to 50 μg./ml. This second set was then placed in the bath at 27° and, after 20 hr., the concentration of material in solution increased from 50 to 170 μg./ml. Prolonged incubation, up to 42 hr., caused no further change, whereas incubation for 6 hr. or less was insufficient for equilibration.

Fig. 1 shows the apparent solubilities of fraction A of neutral-salt-soluble collagen in the temperature range 27–37°. Incubations of at least 20 hr. were used and the fair agreement between samples directly incubated at a given temperature and corresponding ones first brought to 37° for 75 min., indicates that equilibria were attained. Samples whose final temperature was 37° were incubated only at 37° for 75 min. A separate study confirmed the earlier conclusion (Fessler, 1960) that after 75 min. little further change occurred in the concentration of material in solution at 37°.

A set of samples was made of volumes 0·2, 0·33, and 0·5 ml. Each sample contained 100 μg. of fraction A in solution at 4°. The total amount of collagen in solution at 37° was determined for each sample and is shown in Fig. 2. For a constant amount of total material the apparent solubility of fraction A was thus independent of the volume of solvent at 37°.

Sets of samples were made, each of volume 0·5 ml. Samples initially contained 50–250 μg. of fraction A

Table 1. *Fibre formation of a mixture of fraction A and fraction B*

See text for experimental details.

	Concn. of material in supernatant after fibre formation at 37° (μg./ml.)		
	Expt. 1	Expt. 2	Mean
Observed	480	500	490
Calculated for fibres to be composed of the following starting materials			
92% of (A+B)	205	205	205
83% of (A+B)	375	385	380
92% of (A)	595	675	635
100% of (A)	475	560	517
92% of (A+0·2 B)	480	—	—
92% of (A+0·31 B)	—	500	—

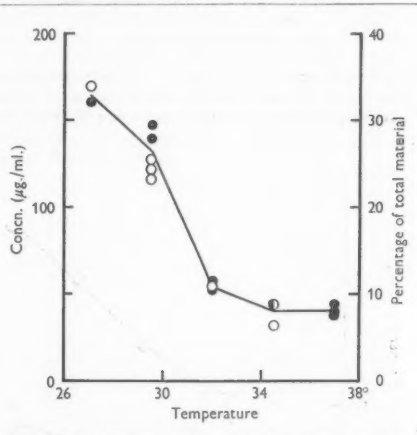

Fig. 1. Effect of temperature on the concentration of collagen in solution. Each sample was initially a solution of 250 μg. of fraction A in 0·5 ml. of 0·067 M-phosphate buffer, pH 6·8, at 2–4°. ○, Results on samples taken directly to the temperature indicated on the abscissa; ●, results on samples first incubated at 37° for 75 min. and then at the temperature indicated on the abscissa. The left-hand ordinates represent the concentration of collagen in solution after incubation for 20 hr. or longer. The right-hand scale of ordinate gives the corresponding percentage of starting material in solution. Each point represents a measurement in duplicate of one sample.

in solution at 4°. The concentrations of collagen in solution at 29·5° and 37° were determined and are shown in Fig. 3. Thus for a constant volume of solvent, the apparent solubility of collagen varied directly with the total amount of collagen present. A line fitted by the method of least squares to the results at 37° and initial concentrations greater than 150 μg./ml. of Fig. 3 has an intercept not significantly greater than zero on the axis. Whether or not the curves of Fig. 3 have a 'shelf' at low initial concentrations could not be determined because of experimental errors, which became appreciable in this range. Definite precipitates were always obtained.

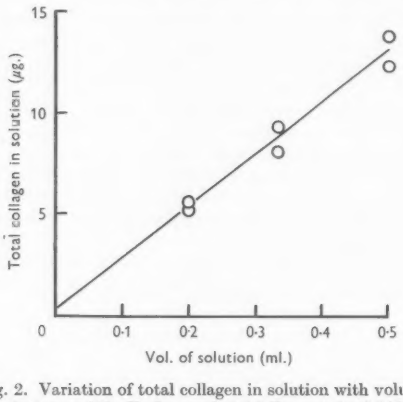

Fig. 2. Variation of total collagen in solution with volume of solution at 37°. Each sample initially contained 100 μg. of fraction A in 0·067 M-phosphate buffer, pH 6·8, at 2–4°.

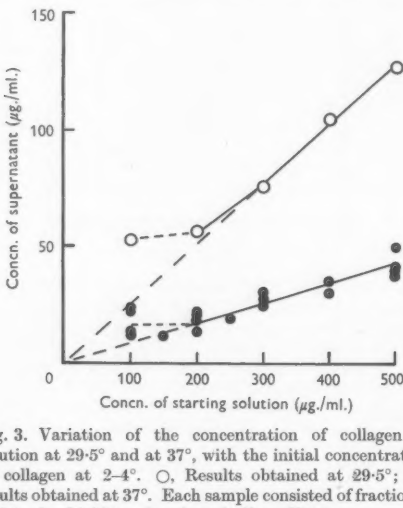

Fig. 3. Variation of the concentration of collagen in solution at 29·5° and at 37°, with the initial concentration of collagen at 2–4°. ○, Results obtained at 29·5°; ●, results obtained at 37°. Each sample consisted of fraction A in 0·5 ml. of 0·067 M-phosphate buffer, pH 6·8.

Polarimetric investigations

On warming a solution of neutral-salt-soluble collagen in 0·067 M-phosphate buffer, pH 7·0, from 0° to 25°, the specific optical rotation of −377° did not change; at higher temperatures fibre formation became appreciable. It has been shown (Fessler, 1960) that the optical rotary power of neutral-salt-soluble collagen is not affected by fibre formation at 37°, followed by careful re-solution. Fig. 4 shows the similar results obtained at different temperatures with solutions of neutral-salt-soluble collagen and acid-soluble collagen, both in 0·067 M-acetate buffer, pH 3·7. At 0° the specific optical rotation of neutral-salt-soluble collagen was the same at pH 3·7 as at pH 7·4: −377°; that of acid-soluble collagen was practically the same: −382°. Warming to 34° or 37° caused a small change of about +13° of arc in both materials and the initial values were restored on cooling. Warming to 39·5° caused a rapid change in both materials, which was hastened at 40°, the final specific rotations at 40° being practically identical (−144° for neutral-salt-soluble collagen and −142° for acid-soluble collagen). This change was partly reversed on

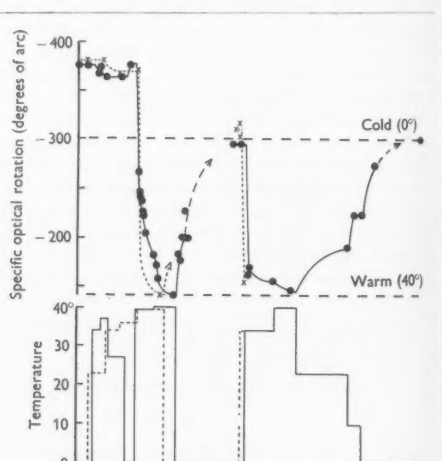

Fig. 4. Effect of various temperatures on the specific optical rotations of neutral-salt-soluble collagen and acid-soluble collagen in 0·067 M-acetate buffer, pH 3·7. The specific optical rotation of a sample shown at a given point in the upper curves was measured when the sample was at a temperature shown by the corresponding ordinate given in the lower curves. ●——●, Neutral-salt-soluble collagen; ×---×, acid-soluble collagen.

cooling and after overnight storage at 2–4° the corresponding rotations were −297° and −309°. Further warming and cooling reproduced the changes between about −143° and −305°, but the initial higher specific rotation could not be restored. The final solutions were dialysed against 0·067 M-phosphate buffer, pH 7·0, in the cold. On warming to 37° these solutions remained clear, and the concentration of material in solution was not altered by subsequent centrifuging at 35° (Spinco rotor no. 40, 20 000 rev./min. for 20 min.).

The specific optical rotation in 0·067 M-phosphate buffer, pH 7·4, at 5° of fraction A was −369° and of fraction C −363°, not differing significantly from that of the parent material. Under these conditions separate preparations of fraction B gave −353° and −332°, which on warming changed to −247° and −245° respectively at 37°. All solutions of fraction B were dilute and so there may be considerable error in the values of the stated specific rotations of fraction B. The variation of rotation with temperature was, however, quite definite for fraction B, both at pH 3·7 and pH 7·0, and the ratio (rotary power at 4°)/(rotary power at 37°) was found to be: 1·28, 1·35, 1·43; mean 1·36. The variation of rotation with temperature of fraction B is sufficient to account for the reversible small change of rotation, some 13° of arc (Fig. 4), which occurred on warming unfractionated neutral-salt-soluble collagen to 37°.

Relationship of fractions A, B and C as indicated by incorporation of [^{14}C]*glycine*

A relationship between newly formed collagen and the soluble collagens has been shown to be likely by Harkness, Marko, Muir & Neuberger (1954), Gross (1958), Jackson (1957) and Orekhovitch (1952); and, in contrast, mature collagen appears normally to be insoluble, or soluble only with difficulty. By comparison, the different solubility properties of fractions A, B and C could reflect different stages of maturation of neutral-salt-soluble collagen. To investigate this possibility [^{14}C]glycine was injected into guinea pigs and 6 hr. later neutral-salt-soluble collagen was isolated from their skins, separated into fractions A, B and C, and the specific radioactivities of the constituent glycine were determined; counts/min. were plotted against concentration of DNP-glycine (spectrophotometer reading) and values for all samples and their dilutions fell on a common straight line. The standard deviation of the slope of this line was in one experiment 2% of the slope of the line, and in the duplicate set 5%. Thus no difference was found in the specific radioactivities of glycine isolated from fractions A, B and C of neutral-salt-soluble collagen 6 hr. after injection of [^{14}C]glycine.

Some observations on the rate of precipitation of neutral-salt-soluble collagen

'Precipitation time' varied inversely as the hydrogen-ion concentration, as shown in Fig. 5. The proportionality coefficient was found to be different in two samples. Below 10° a solution at pH 7·4 had not formed a precipitate after several months, a solution at pH 6·8 would give one after several weeks and one of pH 6·4–6·6 within a few

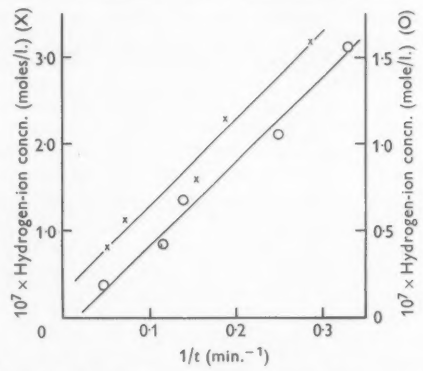

Fig. 5. Variation of 'precipitation time' of neutral-salt-soluble collagen with hydrogen-ion concentration. Abscissae denote reciprocals of 'precipitation times'. Left-hand scale of ordinate denotes hydrogen-ion concentrations for sample (×) of collagen preparation III, and correspondingly the right-hand scale of ordinate for sample (○) of collagen preparation VI. Measurements were made at 30° in 0·067 M-phosphate buffer.

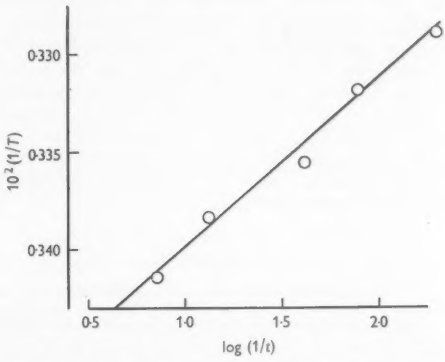

Fig. 6. Variation of 'precipitation time' with temperature. Ordinates are reciprocals of the absolute temperatures, abscissae are logarithms of the reciprocals of 'precipitation times'. Measurements were made in 0·067 M-phosphate buffer, pH 6·8, in the temperature range 20–31°.

Table 2. *Effect of some buffer ions on 'precipitation time'*

To 0·05 ml. of a solution of neutral-salt-soluble collagen in 0·067 M-phosphate buffer, pH 6·8, 0·02 ml. volumes of the following 0·1 M solutions, adjusted to pH 6·8 (glass electrode), were added and the 'precipitation time' was measured at 29·4°.

Substance	'Precipitation time' (min.)
Phosphate	5·7
α-Picoline	2·7
Glyoxaline	3·1
Collidine	3·3

Similar experiment with another sample of neutral-salt-soluble collagen; pH 7·3 throughout.

Substance	'Precipitation time' (min.)
Phosphate	6·5
Tris	2·0

days. A solution at pH 6·8 would form a precipitate within several hours at room temperature and the variation of 'precipitation time' from 20° to 31° is given in Fig. 6. At 37° precipitates were obtained within a few minutes. The slower the rate of precipitation, the more flocculent and easily teased apart was the material and the larger were the fibres formed. Artificial partial alignment of the particles by shearing between concentric cylinders at 180 sec.$^{-1}$, for 6 hr. at 4°, did not cause precipitation. 'Precipitation time' was increased by dilution. At constant final concentration of neutral-salt-soluble collagen, 'precipitation time' was not affected by addition of: (i) the precipitate obtained by warming another sample of neutral-salt-soluble collagen to 37°; (ii) the supernatant of (i); (iii) a dialysed solution of the material left in the skin extracts after removal of the soluble collagen. Table 2 shows the effect of some buffer ions on 'precipitation time'. 'Precipitation times' varied from sample to sample and also depended on the observer. All solutions whose 'precipitation times' were to be compared were included in any one experiment. Repeat experiments led to identical conclusions, though corresponding 'precipitation times' were not necessarily identical; e.g. results given in Fig. 5.

DISCUSSION

Solubility properties of fraction A

The equilibria which were reached were probably independent of the diameter of the precipitated fibres, because slowly formed flocculent precipitates tend to consist of larger fibres than those present in rapidly formed gels, and the nature of the precipitate did not affect the equilibria. This gives some support for assuming the fibrous precipitate to be a single thermodynamic phase. Only with this assumption can the Phase Rule be applied to the finding that the solubility varies with the total amount of fraction A present (Figs. 2 and 3) and it follows that fraction A contains more than one component.

The considerable variation of apparent solubility with temperature (Fig. 1) invites comparison with temperature-dependent properties of gelatin (Ferry, 1948; Robinson, 1953), and this is done in Fig. 7. Specific optical rotations of gelatin obtained from Fig. 4 are plotted at different temperatures, together with the sum, $-[300x + 144(1-x)]$, where x equals the fraction of collagen in solutions at that temperature (from Fig. 1, right-hand scale of ordinate), -300 is the specific optical rotation of gelatin at 0° and -144 that at 40° (from Fig. 4). The similarity suggests that the two phenomena are expressions of one property of the collagen peptide chain.

One interpretation of the solubility properties of fraction A is that there are two components, a soluble 'cold' form and an insoluble 'warm' form, with distribution of material between the two forms

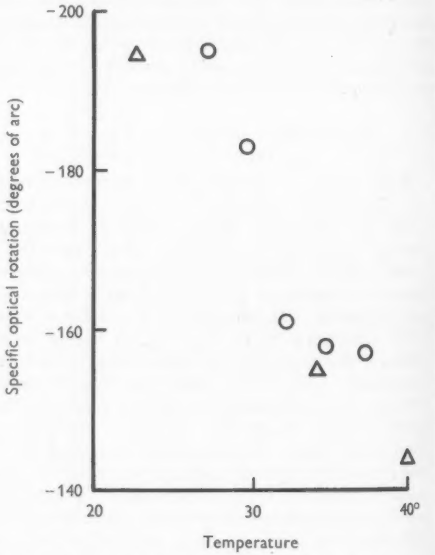

Fig. 7. Comparison of the temperature-dependent behaviour of fraction A of neutral-salt-soluble collagen and of the corresponding gelatin. The specific optical rotations observed for gelatin are denoted by △. Specific optical rotations calculated for gelatin by the method given in the text are shown as ○ and are based on the variation of solubility of fraction A with temperature and the specific optical rotations of gelatin at 0° and 40°.

being regulated only by temperature. Florey (1956) showed theoretically that small variations in interaction energies between solvent and long rod-like particles critically affect the distribution of material among phases. Thus only a small change of the interactions between collagen and solvent with temperature could account for the solubility properties of fraction A. Florey, however, considered only an uncharged solute.

Nature of fraction B

Fraction B could be a mixture of fraction A and gelatin. As fraction B constitutes 16% of the total neutral-salt-soluble collagen (Fessler, 1960) and 8% of fraction A is in solution at 37°, about half of fraction B would be fraction A. From the warming of mixtures of fractions A and B (Table 1), 25% of fraction B is calculated as behaving like fraction A. The specific optical rotations of fraction B at 4° and 37° may be compounded from those of gelatin (−300° at 4°, −144° at 40°) and of the other neutral-salt-soluble-collagen fractions (mean −370°, independently of temperature). This indicates 55% of fraction B to be gelatin.

The behaviour of fraction B at 37° and neutral pH is not in accord with this view: fibres should be formed from that part which is fraction A, but no fibres appear in 0·067M-phosphate buffer. Further, on dialysis against water fraction B was quantitatively precipitated, and, whereas this would be possible if some of fraction B were gelatin, it would not be likely.

Temperature-dependent properties of neutral-salt-soluble collagen

The re-dissolving of fibres of fraction A on lowering the temperature from 37° shows that collagen can be in a stable state in solution. Conversely, fibres form a stable state at 37°, as further warming of fibre–solution systems does not decrease the amount of material in the solid state (unless gelatin is formed). That reversible equilibria can be established is proven by the experiments on fraction A (Fig. 1). At acid pH the fibrous state is probably unstable, as no precipitate appeared on warming solutions of pH 4·5 or less.

The rate of change of collagen from solution to fibre depends on concentration, temperature, pH and the nature of other materials present in solution. The effect of concentration was not in accord with any simple kinetic scheme. From Fig. 6 an activation energy of approx. 80 kcal./mole/degree was calculated by the Arrhenius Equation, which is in the range of values found for protein denaturation. The effects of increasing hydrogen-ion concentration (see Results section and Fig. 5) show that either these ions act catalytically, or the state of ionization of the collagen particle is a rate-determining factor, or both. The influence of a variety of materials added to solutions of collagen has been studied, while this work was in progress, by Gross & Kirk (1958), and Bensusan & Hoyt (1958).

The positively charged, nitrogenous, buffer ions which accelerated the rate of precipitation of neutral-salt-soluble collagen could either act catalytically, or could alter the charge on collagen particles. Interaction of collagen must occur with 2-amino-2-hydroxymethylpropane-1:3-diol (tris) ions, as on dialysis at 2–4° against this buffer (pH 7·3, 0·067M) neutral-salt-soluble collagen was precipitated quantitatively from solution, as it was on warming to 37°. Binding of buffer ions by collagen is well known (Gustavson, 1956; Jackson & Neuberger, 1957). These effects of nitrogenous buffer ions and of hydrogen ions may together indicate that the rate of precipitation is increased with decreasing negative charge on the collagen particle. When there is a net positive charge on collagen, as at pH 3·7 (Jackson & Neuberger, 1957), no fibres are formed on warming a solution. Probably, fibre formation is facilitated when the net charge of the collagen particle is small. In accord with this is the transient precipitate formed when solutions of neutral-salt-soluble collagen are dialysed at 2–4° from 0·067M-phosphate buffer, pH 7·0, into 0·067M acetate buffer, pH 3·7, or vice versa.

The dissolution of fibres on cooling was not studied in detail. This process is accelerated at 2–4° from 48 hr. (Fessler, 1960) to a few hours by dialysing at the same time against 1% acetic acid (or 0·067M-acetate buffer, pH 3·7), thus supporting the previous conclusion that a net positive charge on the particles is contrary to fibre stability.

That gelatin had rapidly been formed at 39·5°, pH 3·7, was shown by the subsequent mutarotation (cf. Ferry, 1948) and the inability of the residual material to form fibres at neutral pH. Although in solution no change was observed with temperature in the optical rotary power of that collagen which could form fibres, it cannot be decided whether the actual fibre formation corresponds to a change in specific optical rotation or not.

Relationship of fractions A, B and C

Although fractions A, B and C had the same rate of incorporation of [^{14}C]glycine over a 6 hr. interval, the fractions could have differed at other times. This, however, is unlikely, for the chances are small that an interval was used such that the specific activity–time curves of materials standing in sequential relationship might overlap sufficiently for separate specific activities to be indistinguishable. Thus no difference was found in the age of the three fractions.

Fractions B and C could be artifacts of preparation or of the first warming to 37°. This appears unlikely in view of (1) there being no difference between materials which had been salt-precipitated three and four times, (2) repeated warming and cooling produces neither fraction B nor fraction C. In view of the diverse effects of substances on the rate of fibre formation (Gross & Kirk, 1958) traces of impurities might affect the equilibria between fibre and solution. At higher ionic strengths such interference would be decreased and this would fit in with the fibre formation of fraction B under these conditions.

General

There are small, yet definite, differences between the neutral-salt-soluble collagen studied and soluble collagen extracted at acid pH. Thus the present material was obtained by repeatedly extracting skins with phosphate buffer at neutral pH. After no more collagen could be dissolved this way, extraction with 0·067 M-citrate, pH 3·6, gave an ample yield of collagen. Subsequent dialysis of this citrate solution of collagen against the phosphate buffer used for previous extractions precipitated the collagen. Conversely some apparent solutions of neutral-salt-soluble collagen in 0·067 M-citrate buffer, pH 3·6, proved to be suspensions in the ultracentrifuge (Fessler, 1960). Further, the rates of incorporation of [^{14}C]glycine *in vivo* into collagens extracted in these two ways differ (Harkness *et al.* 1954; Jackson, 1957). Neutral-salt-soluble collagen and citrate-extracted collagen have closely similar structures, as they are indistinguishable both by optical rotation and the temperature at which rapid conversion into gelatin occurs. The amino acid compositions of these two collagen preparations have been compared by Jackson, Leach & Jacobs (1958). Differences in solubility may not be important, especially as collagen is known to adsorb ions (e.g. Gustavson, 1956; Jackson & Neuberger, 1957). On the other hand, small differences in the interaction energies between collagen particles and solvent may, in theory (Florey, 1956), profoundly affect the distribution between phases and therefore be critical in fibrogenesis. Different impurities in the two preparations could further affect the systems.

The similarity between the effects of temperature upon the solubility behaviour of fraction A and the specific optical rotation of gelatin may reflect an inherent property of the collagen polypeptide chain, in one case constrained and in the other free. Changes in the hydrogen-bonding of gelatin are known to occur with changes of temperature (e.g. Robinson, 1953), and corresponding changes may be principally responsible for the temperature-dependent-solubility behaviour of fraction A.

SUMMARY

1. Fibres formed from concentrated solutions of fraction B of neutral-salt-soluble collagen by salting-out had the typical striated appearance of collagen fibres in the electron microscope.

2. No fibres were formed on warming enriched solutions of fraction B in 0·067 M-phosphate buffer, pH 7·4, although the collagen concentration was several times as great as concentrations at which fraction A forms fibres under these conditions.

3. Solubility properties of fraction A varied considerably with temperature in the range 27–37°, and were not those of a single Phase-Rule component.

4. At pH 3·7 the specific optical rotation of fraction B varied with temperature (0–37°), whereas those of fractions A and C did not.

5. The polarimetric behaviour of solutions of neutral and acid-soluble collagen were identical on varying the temperature at pH 3·7. Conversion into gelatin was rapid at 39·5°.

6. The glycine of fractions A, B and C had identical specific radioactivities in samples isolated from guinea pigs 6 hr. after injection of [^{14}C]glycine.

7. The rate of precipitation of neutral-salt-soluble collagen varied directly with hydrogen-ion concentration in the range pH 6·5–7·4. The activation energy was about 80 kcal./mole/degree at pH 6·8.

8. The temperature-dependent-solubility behaviour of neutral-salt-soluble collagen and the nature of fractions A, B and C are discussed.

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REFERENCES

Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
Ferry, J. D. (1948). *Advanc. Protein Chem.* **4**, 1.
Fessler, J. H. (1960). *Biochem. J.* **76**, 452.
Florey, P. J. (1956). *Proc. Roy. Soc.* A, **234**, 73.
Gross, J. (1958). *J. exp. Med.* **107**, 265.
Gross, J. & Kirk, D. (1958). *J. biol. Chem.* **233**, 355.
Gustavson, K. H. (1956). In *The Chemistry and Reactivity of Collagen*. chapt. 4, 5. New York: Academic Press Inc.

Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.
Jackson, D. S. (1957). *Biochem. J.* **65**, 277.
Jackson, D. S., Leach, A. A. & Jacobs, S. (1958). *Biochim. biophys. Acta*, **27**, 418.
Jackson, D. S. & Neuberger, A. (1957). *Biochim. biophys. Acta*, **26**, 638.
Kroll, S. (1952). *Biochem. J.* **52**, 227.
Orekhovitch, V. N. (1952). *Comm. 2nd int. Congr. Biochem., Paris*, p. 106.
Robinson, C. (1953). In *Nature and Structure of Collagen*, p. 96. Ed. by Randall, J. T. London: Butterworths Scientific Publications.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

Biochem. J. (1960) **76**, 471

The Phospholipids of Human-Blood Plasma and their Exchange with the Cells

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When whole human blood is incubated *in vitro* with radioactive acetate, radioactivity is incorporated into the phospholipids (James, Lovelock & Webb, 1957; Rowe, 1959). Moreover, there is an exchange of the radioactive phospholipids between the cells and the plasma (Lovelock, James & Rowe, 1960). Further information, both on the composition and structure of the plasma phospholipids and their exchange with the cells, is required. This paper reports the analysis of the radioactive phospholipids of a plasma fraction obtained from blood which had been incubated with sodium [*Me*-^{14}C]-acetate. The plasma had been treated with dextran sulphate and the precipitated β-lipoproteins removed before analysis (Oncley, Walton & Cornwell, 1957).

METHODS

The incubation of blood and other experimental procedures are essentially those described by Rowe (1959).

Preparation of the plasma fraction. Whole blood (140 ml.) which had been incubated with sodium [*Me*-^{14}C]acetate (30 μc; specific activity 13·7 mc/mg.) and [^{32}P]orthophosphate (1·6 mc; carrier-free) for 6·5 hr. was centrifuged at 600 *g* for 5 min. The cells were removed and calcium chloride (2 ml., 0·25 M) and dextran sulphate (6 ml.; 1%, w/v) were added to the stirred plasma. After 20 min. the plasma was centrifuged again at 600 *g* to remove the precipitated β-lipoprotein (Oncley *et al.* 1957). A previous experiment established that under these conditions all of the red and white cells, and most of the platelets, were removed from the plasma by the first centrifuging and that the remaining platelets were removed with the precipitated β-lipoprotein.

Extraction of phospholipids. The supernatant plasma was added to 2 l. of ethanol–ether (2:1, v/v). The mixture was allowed to stand at room temperature for 24 hr. and then stored at 2° in the dark for 3 weeks. The solids were removed by filtration and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator. The residue was extracted twice with 10 ml. of acetone and then successively with 10, 20 and 10 ml. of ethanol. All extractions were performed at room temperature. The small amount of insoluble residue in the ethanol extract was removed by centrifuging. The β-lipoprotein–dextran sulphate complex was added to ethanol–ether (500 ml.) and treated in the same manner.

Chromatography of phospholipids. Ethanol extract (35 ml.) from the β-lipoprotein-free plasma containing 74·9 μmoles of phosphorus was distilled to dryness under reduced pressure and the residue suspended in 150 ml. of chloroform–methanol (7:1, v/v). The suspension was passed through a column of silica gel (2·5 cm. diam. × 33 cm.). The silica gel was prepared by the method of Lea, Rhodes & Stoll (1955) and the column constructed as described by Hannahan, Dittmer & Warashina (1957). The column was then washed successively with chloroform–methanol mixtures of volume ratios 7:1 (235 ml.), 4:1 (270 ml.), 3:2 (295 ml.) and 1:4 (290 ml.). The flow rate was approx. 0·9 ml./min. Eluate fractions of approx. 4 ml. were collected.

Estimation of radioactivity. Each eluate fraction was diluted to 5 ml. with its own solvent mixture and 0·2 ml. evaporated to dryness on a polythene planchet. The radioactivity of each fraction was estimated by counting with an end-window Geiger–Müller tube. The fractions were combined according to their radioactive peaks, as shown in Fig. 1. The combined fractions were concentrated and then diluted to 10 ml. or 25 ml. with chloroform and methanol. Portions (0·20 ml.) containing 0·18–2·31 μmole of phosphorus were concentrated to dryness on planchets and their radioactivities estimated. The radioactivity due to ^{32}P and ^{14}C in the unfractionated phospholipids (Table 1) was estimated in the same way with 0·10 ml. of solution. The planchet was then shielded with a foil of calibrated phosphor-bronze and the radioactivity due to ^{32}P estimated (Rowe, 1959).

Identification of the fractions. The fractions were chromatographed on silica-impregnated paper with 20% methanol in chloroform (Lea *et al.* 1955). The components were detected by immersion of the chromatogram in iodine

vapour and were identified by their R_F values. On separate chromatograms the kephalins were detected by spraying with ninhydrin and heating at 110° for 5 min. The choline-containing lipids were detected by treatment with phosphomolybdic acid followed by reduction with stannous chloride (Chargaff, Levine & Green, 1948).

Identification of the fatty acids. The fractions were subjected to methanolysis by heating in anhydrous methanolic 7% hydrogen chloride at 110° in a sealed tube (Rowe, 1959). After neutralization with silver carbonate the methyl esters of the fatty acids were extracted with light petroleum and analysed by gas chromatography by using Celite covered with an adipic acid–ethylene glycol-condensation resin (Lipsky & Landowne, 1958).

Test for haemolytic activity of phospholipid fractions. Freshly drawn blood (about 0·5 ml.), mixed with citric acid–sodium citrate–dextrose solution (0·1 ml.; 0·83 M-trisodium citrate, 0·35 M-citric acid and 0·1 M-glucose), was centrifuged to remove the plasma. The cells were washed three times with an equal volume of 0·89% sodium chloride solution and then suspended in approximately nine times their volume of 0·89% sodium chloride. The phospholipid fraction, containing 0·05 μmole of phosphorus, was concentrated to dryness and suspended in 0·5 ml. of 0·89% sodium chloride. Cell suspension (0·5 ml.) was added and the mixture allowed to stand, with intermittent shaking, for 1 hr. at 36°. The mixture was centrifuged and the supernatant fluid examined for haemoglobin.

RESULTS AND DISCUSSION

The yield, specific radioactivity and total radioactivity of the unfractionated phospholipids from the β-lipoprotein-free plasma are shown in Table 1. The corresponding values for the β-lipoprotein and the cells are shown for comparison. The fraction from the β-lipoprotein, which was comparatively low in yield and likely to be contaminated by phospholipids from platelets and chylomicra, was not examined further. The ^{32}P specific activity of the phospholipids from the β-lipoprotein-free plasma was comparatively low.

The eluate fractions from chromatography were counted at a period equivalent to ten ^{32}P half-lives after the incubation of whole blood, and the radioactivity due to ^{32}P was therefore negligible. These radioactivities are shown in Fig. 1. The way in which these fractions were combined is also shown. The yields, compositions and radioactivities of the combined fractions are shown in Table 2. The distributions of the fatty acids in each fraction are given in Table 3. A comparison of the yields and radioactivities of the plasma phospholipids with those of the combined phospholipids from the cells and platelets from the same experiment are shown in Table 4. The analysis of the latter has been reported in detail by Rowe (1959). A comparison of the fatty acid compositions of the combined cellular and platelet lecithin with the plasma-fraction lecithin is given in Table 5. The same comparison is given for sphingomyelin.

The phosphorus recovery from the silica-gel column was 100%. The ratios sphingomyelin:lecithin:kephalin expressed as moles of phosphorus were 1·0:6·9:0·8. Phillips (1958), who analysed whole plasma, obtained the ratios 1·0:3·6:0·24 respectively. The difference between these sets of values probably reflects the phospholipid composition of the precipitated β-lipoprotein. Fractions 11 and 12 contained a choline-containing component which when chromatographed on silica-impregnated paper had an R_F (0·27) expected for lysolecithin. These fractions produced some haemolysis in a suspension of human red blood cells, which is consistent with their containing lysolecithin. It is uncertain whether the lysolecithin was present in the plasma or arose from the decomposition of a labile component, e.g. choline plasmalogen, during chromatography. Fractions 2, 4, 5, 6, 8, 9 and 13 when tested showed no haemolytic activity.

Radioactive kephalin, lecithin and sphingomyelin were all found in the plasma. An unknown fraction (fraction 1) of high specific activity was also isolated. This fraction, in which neither choline nor ninhydrin-staining components were detected, contained fatty acids (Table 2). On chromatography on silica-impregnated paper it contained a well-defined component which stained heavily with iodine vapour. This had an R_F (0·94) greater than that of kephalin (0·85).

The cells in human blood which might be expected to synthesize phospholipids include erythrocytes, reticulocytes, leucocytes and platelets. In this Discussion these are called collectively cells. Some or all of these might be expected to synthesize phospholipids and exchange with each other or with the plasma. In such a complex system as human blood it is difficult to distinguish between synthesis and exchange. In this analysis, however, the radioactivity is contained in lipids

Table 1. *Yields and specific activities of phospholipids from human-blood fractions*

		Cells	β-Lipoprotein-free plasma	β-Lipoprotein
Yield (μmoles)		190	107	22·3
Specific activity (counts/min./μmole)	^{32}P	3 200	139	936
	^{14}C	256	17·4	130
Total activity (counts/min.)	^{32}P	608 000	14 880	20 860
	^{14}C	48 300	8 270	2 900

synthesized by the cells and excreted into the plasma. Previous experiments indicated (Lovelock *et al.* 1960) that the specific activities of the cellular phospholipid fatty acids rise over 5 hr. and then remain constant for the next 15 hr. An incubation time of 6·5 hr. was therefore chosen for the present experiment.

The specific radioactivities of the plasma lecithin and sphingomyelin are higher than those of the cellular lecithin and sphingomyelin respectively.

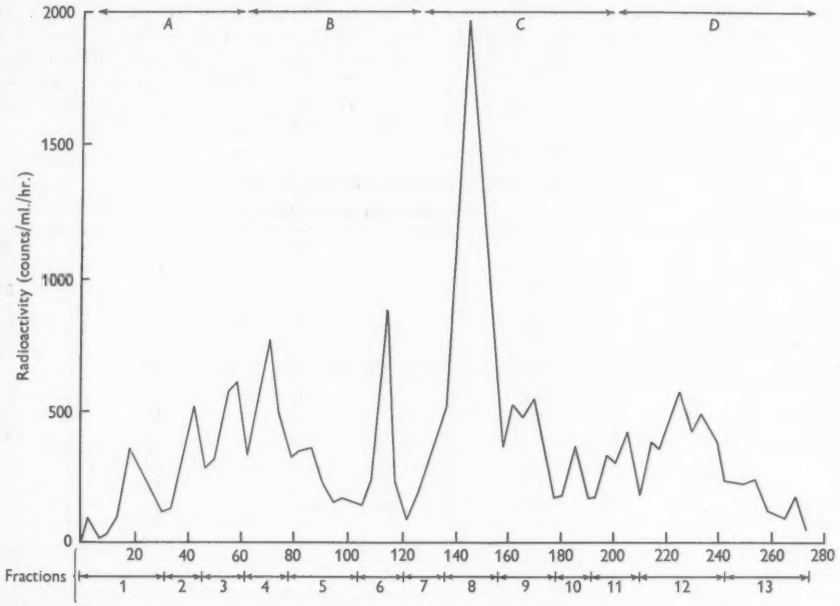

Fig. 1. Chromatography on a column of silica gel of phospholipids from human plasma. The upper row of numbers on the abscissa indicates the number of fractions (approx. 4 ml.) collected. The lower row of numbers indicates how these fractions were grouped. Elution solvent was chloroform–methanol mixtures of the following proportions (v/v): *A*, 7:1; *B*, 4:1; *C*, 3:2; *D*, 1:4.

Table 2. *Chromatographic analysis of plasma phospholipids*

Total analysed = 74·9 μmoles of P. 'Unknown' refers to a ninhydrin-staining component extending from origin.

Fraction from column (see Fig. 1)	Yield (μmoles of P)	Identification	Specific activity (counts/min./ μmole of P)	Yield × specific activity × 10^{-3}
1	1·69	—	1620	2·74
2	0·89	—	1860	1·66
3	2·23	Kephalin	510	1·14
4	2·01	Kephalin	28·6	0·57
5	28·82	Lecithin	104	3·06
6	5·55	Lecithin	207	1·15
7	4·00	Lecithin	485	1·94
8	8·45	Sphingomyelin + lecithin	798	6·74
9	4·73	Sphingomyelin + unknown (trace)	783	3·70
10	3·16	Sphingomyelin + unknown	463	1·46
11	7·90	Lysolecithin + unknown + sphingomyelin	502	3·96
12	2·10	Lysolecithin + unknown	162	0·34
13	3·25	Unknown + lysolecithin (trace)	246	0·80
Total	74·8			

This suggests that only a fraction of these cellular phospholipids is involved in biosynthesis and exchange. Further information is available from consideration of the distributions of fatty acids. Previous experiments (Lovelock *et al.* 1960) indicated that in whole blood at 34·5° in the presence of excess of acetate, phospholipid exchanged between cells and plasma with a half-life of approximately 6 hr. Exchange of all the blood phospholipid would be expected to result in similar distributions of fatty acids in plasma and cells. This is clearly not the case. Cellular lecithin contained arachidonic acid not found in the plasma lecithin (Table 5). Moreover, there were large differences between plasma and cellular lecithin in the percentages of palmitic acid, stearic acid, oleic acid and linoleic acid. Thus it would appear that for lecithin at least only a fraction of the cellular and plasma phospholipids are involved in exchange.

Recent investigations (Rowe, Allison & Lovelock, 1960) have shown that leucocytes are responsible for most of the incorporation of acetate into the cellular phospholipids. It is likely therefore that it is the leucocyte phospholipids that are involved in exchange with the plasma.

Table 3. *Fatty acids of plasma-phospholipid fractions*

	Fractions from column (see Fig. 1)									
Acid	1	4	5	6	7	8	9	10	11	13
Lauric	0·9	1·6	5·2	0·8	—	3·2	6·2	1·1	0·5	1·2
Unknown	0·9	1·2	0·3	0·6	—	—	15·6	1·0	—	0·9
Unknown	—	—	—	—	—	—	4·0	—	—	—
Myristic	4·9	4·0	2·9	1·9	2·0	5·4	16·3	4·2	2·2	4·6
Unknown	—	—	0·7	—	—	—	—	—	1·0	—
Unknown	—	3·6	0·4	1·7	0·8	—	—	1·0	0·9	—
Unknown	—	5·6	0·7	1·6	—	—	—	—	0·7	—
Palmitic	28·6	21·1	36·8	44·0	55·1	56·0	31·5	62·8	6·7	5·7
Palmitoleic	4·3	6·7	2·1	3·5	—	—	—	1·2	2·9	8·2
Unknown	—	4·0	0·5	1·2	1·6	—	—	—	0·1	2·4
Unknown	—	7·1	—	—	—	—	—	—	1·5	—
Stearic	8·0	16·6	14·8	10·3	9·5	11·6	26·4	10·7	11·1	13·3
Oleic	35·0	11·5	12·2	13·0	17·7	11·6	—	5·2	6·9	14·4
Linoleic	11·8	6·7	16·3	16·1	13·1	12·3	—	3·7	3·4	6·0
Linolenic	5·4	—	0·6	—	—	—	—	3·2	6·3	19·6
Unknown	—	—	2·3	2·2	—	—	—	5·4	5·9	—
Unknown	—	—	4·4	3·1	—	—	—	—	—	23·4
Arachidonic	—	10·1	—	—	—	—	—	—	—	—

Table 4. *Comparison of radioactivities of cellular and plasma phospholipids*

	Yield* (μmoles)		Specific activity (counts/min./μmole)		Yield × specific activity × 10^{-3}	
Phospholipid	Cells	Plasma	Cells	Plasma	Cells	Plasma
Fraction 1 (see Fig. 1)	0·78	2·58	466	1700	0·36	4·40
Phosphatidylethanolamine	—	—	81; 300	—	—	—
Phosphatidylserine	—	—	800	—	—	—
Total kephalin	33·8	4·2	403	407	13·6	1·71
Lecithin	27·2	38·4	100	160	2·72	6·15
Sphingomyelin	16·0	13·2	160	791	2·55	10·4

* Yield calculated for the same volume of blood.

Table 5. *Comparison of the fatty acid compositions of cellular and plasma lecithin and sphingomyelin*

	Lecithin							Sphingomyelin			
		Plasma			Plasma/cells				Plasma		
Acid	Cells							Cells			
Myristic	2·3	2·9	1·9	2·0	1·3	0·83	0·87	1·5	5·4	16·3	4·2
Palmitic	5·2	36·8	44·0	55·1	7·1	8·5	10·6	30·8	56·0	31·5	62·8
Stearic	2·2	14·8	10·3	9·5	6·7	4·7	4·3	9·6	11·6	26·4	10·7
Oleic	5·0	12·2	13·0	17·7	2·4	2·6	3·5	7·9	11·6	0	5·2
Linoleic	53·4	16·3	16·1	13·1	0·31	0·30	0·25	7·6	12·3	0	3·7
Arachidonic	22·7	0	0	0	0	0	0	0	0	0	0
C_{24} acids	9·2	0	0	0	0	0	0	32·6	0	0	0

SUMMARY

1. The analysis, by silica-gel chromatography, of phospholipids from a human-plasma fraction is described. The fraction was derived from whole blood which had been incubated with sodium [*Me*-^{14}C]acetate for 6·5 hr. The β-lipoproteins had been removed by precipitation with dextran sulphate.

2. All the phospholipids isolated were labelled.

3. The fatty acids of the isolated phospholipids were analysed by vapour-phase chromatography.

4. A comparison of yield, radioactivity and fatty acid distribution with those for the phospholipids of the cells is given.

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REFERENCES

Chargaff, E., Levine, C. & Green, C. J. (1948). *J. biol. Chem.* **175**, 67.

Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.

James, A. T., Lovelock, J. E. & Webb, J. (1957). *Biochem. J.* **66**, 60P.

Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.

Lipsky, S. R. & Landowne, R. A. (1958). *Biochim. biophys. Acta*, **27**, 666.

Lovelock, J. E., James, A. T. & Rowe, C. E. (1960). *Biochem. J.* **74**, 137.

Oncley, J. L., Walton, K. W. & Cornwell, D. G. (1957). *J. Amer. chem. Soc.* **79**, 4666.

Phillips, G. B. (1958). *Biochim. biophys. Acta*, **29**, 594.

Rowe, C. E. (1959). *Biochem. J.* **73**, 438.

Rowe, C. E., Allison, A. C. & Lovelock, J. E. (1960). *Biochim. biophys. Acta*, (in the Press).

Biochem. J. (1960) **76**, 475

Metabolic Heterogeneity of Human γ-Globulin

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Human γ-globulin is known to be heterogeneous according to several immunological and physicochemical criteria. Nevertheless, the labelled protein appears to be eliminated from plasma at a constant rate after the initial phase of equilibration between intra- and extra-vascular pools and this suggests that the molecules have a uniform metabolic fate (Myant, 1952; Dixon, Talmage, Maurer & Deichmiller, 1952; Volwiler *et al.* 1955). Turnover studies based on plasma-elimination data alone cannot, however, detect minor components which are rapidly broken down during the phase of equilibration; nor do such studies constitute a sensitive means of detecting a steady fall in the rate of breakdown such as might occur with a mixed population of molecules.

For assessing the metabolic homogeneity of plasma-protein preparations, radioactive iodine offers advantages over other isotopes. ^{131}I liberated during catabolism is not re-incorporated into protein and, provided that adequate amounts of inactive iodide are administered, the radioactive isotope is quantitatively excreted in the urine (Cohen, Holloway, Matthews & McFarlane, 1956). Daily turnover rates can be calculated by expressing excreted radioactivity as a fraction of the labelled protein remaining in the plasma (McFarlane, 1957). The turnover rate of albumin measured by this method remains constant during 4 weeks of observation in healthy human subjects (S. Cohen, T. Freeman & A. S. McFarlane, unpublished work); turnover rates of γ-globulin, on the other hand, decline progressively both in man and in laboratory animals (McFarlane, 1957).

We have applied this test of metabolic homogeneity to several different preparations of human γ-globulin. The results obtained with fractions prepared by electrophoresis and ion-exchange chromatography, as well as with γ-macroglobulin separated by ultracentrifuging, are described below.

METHODS

Subjects. This study was carried out on fourteen men and one woman (ages 19–76 years). Ten appeared to be in perfect health and were members of the hospital or laboratory staff. The remaining five were patients who had been convalescent for 2–4 weeks before the present investigation was started (Table 1).

Preparation of γ-globulin fractions. γ-Globulin was prepared from the blood of seven different healthy donors by the following methods.

(*a*) Zone electrophoresis. A globulin precipitate was obtained from human serum by adding 26% (w/v) Na_2SO_4 to give a final concentration of 16% of Na_2SO_4; the precipitate was dissolved in 0·9% NaCl solution, dialysed

Table 1. *Experimental data in 15 subjects injected with I-labelled γ-globulin*

Protein fractions were prepared by Na_2SO_4 precipitation and electrophoresis (S.E.), by electrophoresis alone (E), by chromatography (A, B and C) and by ultracentrifuging and electrophoresis (U) (see Methods section). All subjects were males except A16. Normal subjects were members of the hospital or laboratory staff.

					Labelled protein				
Expt. no.	Age (years)	Diagnosis	Body wt. (kg.)	Blood donor	Fractionation	Isotope of I	Radio-activity (μc)	Excretion during initial 24 hr. (% of dose)	Duration of expt. (days)
205A	21	Normal	77	1	S.E.	131	246	6·3	42
205B	19	Normal	68	1	S.E.	131	259	6·4	42
205C	20	Normal	74	1	S.E.	131	257	5·6	30
A10	20	Enuresis	—	2	E	131	82	7·3	18
A11	19	Pneumonia	63	2	E	131	77	7·9	18
A16	39	Cystitis	72	3	A	131	69	5·6	21
A17	43	Normal	109	3	A	131	84	4·1	21
A82	30	Normal	60	4	A	125	25	2·9	21
A23	76	Bronchitis	72	3	B	131	86	7·1	21
A24	42	Coronary thrombosis	71	3	B	131	66	6·8	23
A68	32	Normal	90	5	A	131	83	—	41
				5	B	125	11	—	—
A80	48	Normal	107	4	A	125	38	4·3	24
				4	B	131	22	6·5	—
A85	38	Normal	77	5	A	125	51	2·5	19
				5	C	131	18	3·3	—
A53	28	Normal	77	6	U	131	8	29·0	6
A90	38	Normal	80	7	U	131	24	42·0	8
				4	A	125	24	4·6	10

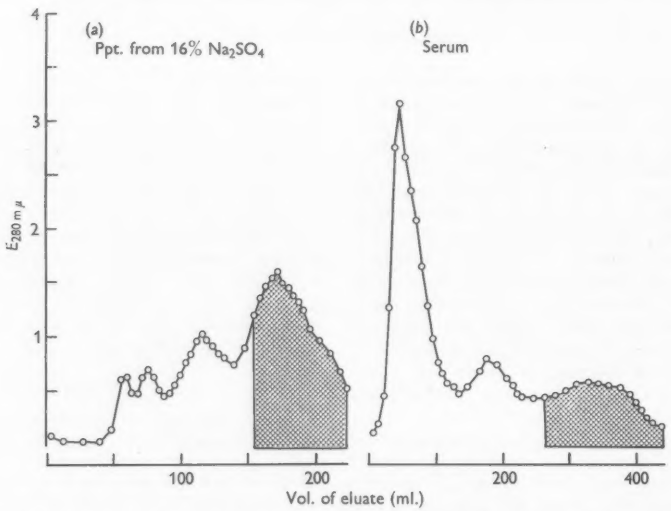

Fig. 1. Zone electrophoresis of a crude globulin precipitate (*a*) and of human serum (*b*). γ-Globulin fractions which were pooled and concentrated by pressure dialysis are shown as shaded areas. See text for experimental details.

against a borate buffer (11·5 mM-$Na_2B_4O_7$–15·5 mM-NaH_2PO_4, pH 8·6) and fractionated by zone electrophoresis on a column of treated cellulose (Porath, 1956). The column measured 40 cm. × 2·5 cm. and was surrounded by a jacket through which tap water was circulated. The eluate was collected in volumes of 4 ml. and protein peaks were located by measurement of the ultraviolet absorption at 280 mμ of volumes (4 ml.) of eluate. The pooled γ-globulin (Fig. 1*a*) was concentrated by pressure dialysis at 4°; this fraction contained 13·6% of the serum protein applied to the column. γ-Globulin was also prepared by zone electrophoresis of 6 ml. of human serum without preliminary salt fractionation and with a cellulose column 83 cm. × 3 cm. This γ-globulin fraction, pooled as shown in Fig. 1*b*, contained 14·9% of the serum protein applied to the column.

(*b*) Chromatography. Diethylaminoethylcellulose was prepared according to the method of Peterson & Sober (1956). A volume of normal human serum (42 ml.) containing 3 g. of protein was decreased to 27 ml. by pressure dialysis against 5 mM-sodium phosphate buffer, pH 7·1; 9% of the protein precipitated during dialysis and the remainder was applied to a column (38 cm. × 2·5 cm.) containing 35 g. of cellulose. The buffer used was 5 mM-phosphate, pH 7·1, with a gradient to 0·3 M-NaH_2PO_4; the volume of the mixing chamber was 1100 ml. The ultraviolet absorption of volumes (4 ml.) of the eluate was measured at 280 mμ and γ-globulin fractions were pooled as shown in Fig. 2*a*. Fraction A contained 7·2% of the protein applied to the column and fraction B contained 1·7%; 75% of the total protein applied to the column was eluted.

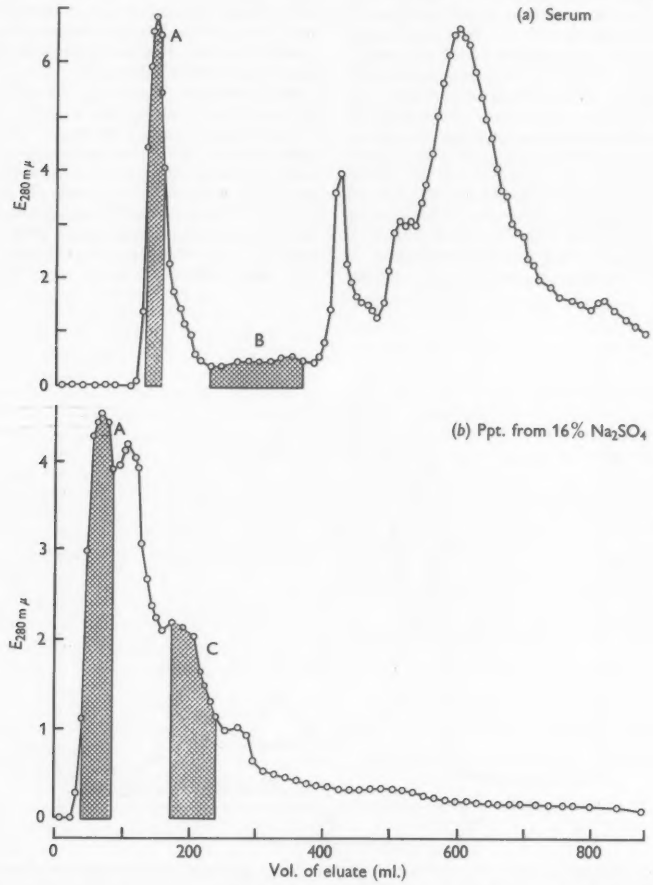

Fig. 2. Chromatography of human serum (*a*) and of globulin precipitated by 16% Na_2SO_4 (*b*). Fractions A, B and C shown as shaded areas were concentrated by pressure dialysis. See text for experimental details.

A globulin precipitate was prepared from normal human serum by precipitating three times in 16% Na_2SO_4. This protein was dissolved in 0·01 M-phosphate buffer, pH 8·3, and dialysed against the same buffer; 2% of the protein precipitated during dialysis and the remainder was applied to a 20 g. diethylaminoethylcellulose column (26 cm. × 2·5 cm.). The buffer was 0·01 M-phosphate, pH 8·3, with a gradient to 0·3 M-NaH_2PO_4; the volume of the mixing chamber was 1100 ml. γ-Globulin fractions were pooled as shown in Fig. 2*b*. Fraction A contained 3·5% of the original serum protein and on rechromatography was identical with fraction A of the previous preparation (Fig. 2*a*); fraction C contained 1·7% of the original serum protein.

(*c*) Ultracentrifuging. Protein solutions, highly enriched in macroglobulin, were prepared from samples (250 ml.) of normal human serum by preparative ultracentrifuging in a Spinco model L ultracentrifuge. The heavy material was spun down eight times at an average centrifugal force of 54 450 *g* for 15 hr. Beginning with the third centrifuging the heavy material was resuspended in 1% of human albumin (Behringwerke, Marburg-Lahn) in an attempt to prevent denaturation (Müller-Eberhard, Kunkel & Franklin, 1956). The final preparation examined in the analytical ultracentrifuge showed one major component having a sedimentation coefficient ($S_{20,w}$) of 17·2 and two minor components having $S_{20,w}$ values of 4·5 (the added albumin) and 24·5; the latter comprised approx. 10% of the total protein. About 10 mg. of the protein prepared by ultracentrifuging was labelled with ^{131}I, mixed with 3 ml. of human serum and separated by zone electrophoresis on a column of treated cellulose (85 cm. × 3 cm.). Labelled albumin, α-macroglobulin and γ-macroglobulin were localized by measurement of radioactivity. The γ-macroglobulin, which contained 14% of the ^{131}I, was pooled as shown in Fig. 3, added to 100 mg. of human albumin (Lister Institute, Elstree, Herts), concentrated by pressure dialysis and passed through a Seitz filter before injection.

Iodination. Protein solutions were labelled with carrier-free ^{131}I or ^{125}I supplied by The Radiochemical Centre, Amersham, Bucks, free from reducing agent and condensed into 0·02 N-NaOH. Iodine monochloride was used as the inactive carrier solution according to the method of McFarlane (1958). Free iodide was removed from labelled protein solutions by passage through anion-exchange columns of De-Acidite (The Permutit Co. Ltd., London, W. 4). After the labelling, 40–60% of the initial radioactivity passed through the column and less than 1·5% of this was present in the supernatant after precipitation with 10% (w/v) trichloroacetic acid. The mean ratio, iodine bound: protein (mol.wt. 150 000) was 1–1·5 g.atom/mole. The labelled proteins were collected in 5% albumin (Lister Institute, Elstree, Herts) and passed through a Seitz filter before injection.

Measurement of radioactivity. ^{131}I and ^{125}I were measured by scintillation counting with a well-type NaI crystal and a pulse-height analyser having a 9 v channel width. Measurements of ^{131}I were made with the channel in the region of the 0·36 Mev γ-ray peak and ^{125}I was measured in the region of the 0·035 Mev peak. In the samples which contained both isotopes ^{125}I measurements were corrected for ^{131}I counts (equivalent to 18·5–20% of the count rate observed in the ^{131}I channel). ^{131}I measurements, on the other hand, did not require correction for ^{125}I, as all

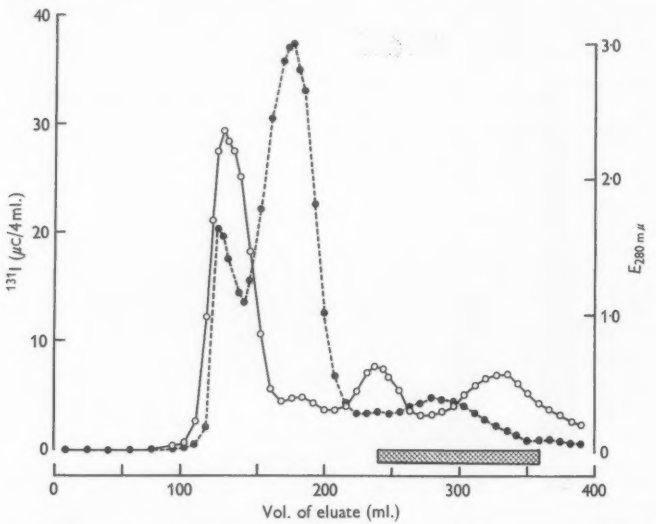

Fig. 3. Zone electrophoresis of a ^{131}I-labelled macroglobulin preparation which contained some added human albumin. About 10 mg. of labelled macroglobulin was mixed with 3 ml. of human serum. The labelled γ-macroglobulin which was concentrated by pressure dialysis is indicated by the shaded area. ●, Radioactivity; ○, extinction.

batches of the latter isotope, when used, gave a negligible counting rate in this channel (less than 0·2 % of the rate observed in the ^{125}I channel). The standard deviation of counts recorded in this study did not exceed ±3 %.

Hexosamine estimations. These were performed by the method of Rondle & Morgan (1955) after hydrolysis of γ-globulin in 4 N-HCl for 4 hr.

Paper electrophoresis. Serum samples were analysed by paper electrophoresis according to the method of Jencks, Jetton & Durrum (1955) and the proportion of γ-globulin was estimated by the elution of strips stained with bromophenol blue. The distribution of radioactivity along strips was determined by scanning before a Geiger counter shielded by lead with a 1·5 mm. window and connected to a ratemeter with pen recorder.

Ultracentrifugal analysis. Solutions containing approx. 10 mg. of protein/ml. were dialysed against 0·15 M-NaCl and examined at room temperature in a Spinco model E ultracentrifuge. Sedimentation coefficients expressed as Svedberg units and corrected to 20° ($S_{20,w}$) were calculated according to the method described by Charlwood (1955).

Experimental procedure. Subjects received NaI (45 mg./day) for 2 days before the injection of labelled γ-globulin and subsequently throughout the period of blood sampling. A volume of 2–5 ml. of labelled γ-globulin (2–8 mg. of protein) was injected into the antecubital vein; residual radioactivity retained in the syringe, needle and ampoule was measured and the dose injected (Table 1) calculated by difference. Blood samples were collected into tubes containing 0·05 ml. of heparin (5000 i.u./ml.). The first was taken 5–10 min. after injection and subsequent samples were collected at intervals of 1–2 days for up to 6 weeks (Table 1). Plasma-protein concentration was determined by a biuret method (Gornall, Bardawill & David, 1949). Radioactivity measurements were made on samples (2 ml.) of plasma and expressed as μc/ml.; during the initial 5 days in which measurements could be made less than 1·5 % of the plasma radioactivity was present in the supernatant after precipitation with trichloroacetic acid at a final concentration of 10 %. Collection of pooled 24 hr. samples of urine was continued for 14–25 days; the radioactivity of samples (2 ml.) was measured. In two experiments (A 10 and A 11) the faeces contained 0·7–0·9 % of the total radioactivity excreted during the first 5 days; faecal analyses were not performed in subsequent experiments.

Analysis of data. The following calculations were made:

Plasma volume =

$$\frac{\text{Injected dose } (\mu c)}{\text{Plasma radioactivity } (\mu c/\text{ml.}) \text{ after 5–10 min.}}$$

Plasma-protein mass = plasma vol. × protein concn.

Plasma-γ-globulin mass = plasma-protein mass × % of γ-globulin (by paper electrophoresis).

Total body radioactivity = injected radioactivity − cumulative urinary excretion of labelled I.

Extravascular radioactivity = total body radioactivity − intravascular radioactivity.

The fractional turnover rate (fraction of the total or intravascular γ-globulin broken down per day) and the mass ratio of extra- to intra-vascular γ-globulin were calculated by the following methods.

(i) From the slope of the plasma specific activity curve (Sterling, 1950).

(ii) By analysis at the 'equilibrium time', i.e. the time at which total activity in the extravascular pool reaches a maximum. As shown by Campbell, Cuthbertson, Matthews & McFarlane (1956) there is no net transfer of radioactivity between intra- and extra-vascular compartments at this time. The rate of change of plasma specific activity at the equilibrium time is therefore equal to the fractional turnover rate and the ratio of intra- to extra-vascular total activities provides a measure of the mass ratio of protein in the two compartments (Fig. 6).

(iii) By resolution of the plasma specific activity curve into two or more exponential functions of time; the rate constants of the system are calculated from the slopes and coefficients of these exponentials (Matthews, 1957). In the present study this analysis was performed by means of an analogue computer in which pool masses and exchange rates are simulated by means of a capacity resistance network (Perkins & Piper, 1959). The plasma specific activity curve was drawn on a graticule in front of the cathode-ray tube and the electrical parameters were adjusted to match the experimental curve. Pool masses were then derived from the relative values of the capacities; the resistance values gave the fractional turnover rate as well as exchange rates between pools.

(iv) Fractional turnover rates were also calculated from the daily urinary excretion of radioactive iodine expressed as a fraction of the mean total intravascular radioactivity during the corresponding period.

RESULTS

γ-Globulin prepared by electrophoresis. γ-Globulin prepared by electrophoresis showed one predominant component in the ultracentrifuge ($S_{20,w}$ 6·75) and about 5 % of heavier material ($S_{20,w}$ 19). The hexosamine content was 1·48 % (Table 2). The ^{131}I-labelled protein was mixed with serum and analysed by paper electrophoresis; radioactivity was confined to the γ-globulin region (Fig. 4). On chromatographic analysis the labelled γ-globulin was widely distributed (Fig. 5); 64 % of the eluted radioactivity emerged with the initial γ-globulin peaks, 25 % was associated with β-globulin and 9 % was eluted beyond the albumin peak in the region of the eluate which contained γ-macroglobulin (see below). The β-globulin area of the chromatogram (Fig. 5, 240–280 ml. of the eluate) was concentrated by pressure dialysis, mixed with unlabelled serum and analysed by paper

Table 2. *Hexosamine content and sedimentation coefficients of γ-globulin preparations*

A, B and C refer to chromatographic fractions (Fig. 2*b*).

γ-Globulin fraction	Hexosamine (%)	Sedimentation coefficient ($S_{20,w}$)
Electrophoresis	1·48	6·75 and 19
A	1·18	6·10–6·40*
B	1·23	6·30–6·62*
C	1·62	6·30

* Range in three separate samples.

electrophoresis; radioactivity was again found to be confined to γ-globulin.

Three subjects received ^{131}I-labelled γ-globulin prepared by sodium sulphate precipitation and electrophoresis; two were injected with material prepared by electrophoresis alone (Table 1). The results were similar in all subjects. Plasma specific activities plotted on a semi-logarithmic scale showed the usual rapid initial decrease associated with mixing of labelled molecules with extravascular protein (Fig. 6*a*). This phase was followed after 7–10 days by an apparently linear rate of decrease; half-lives calculated from this portion of the plasma curve varied from 15 to 21 days (mean 18 days). The apparent mass ratio of extra- to

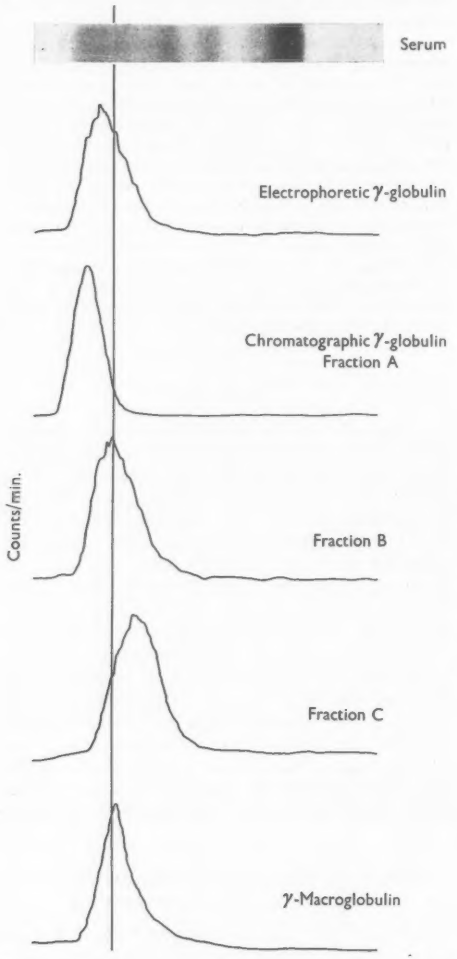

Fig. 4. Electrophoretic analysis and radioactive assay of labelled γ-globulin preparations. A tracer amount of each labelled γ-globulin preparation (see Methods section) was added to normal human serum and analysed by paper electrophoresis. The distribution of radioactivity on the paper strips was determined by scanning before a Geiger counter shielded by lead with a 1·5 mm. window.

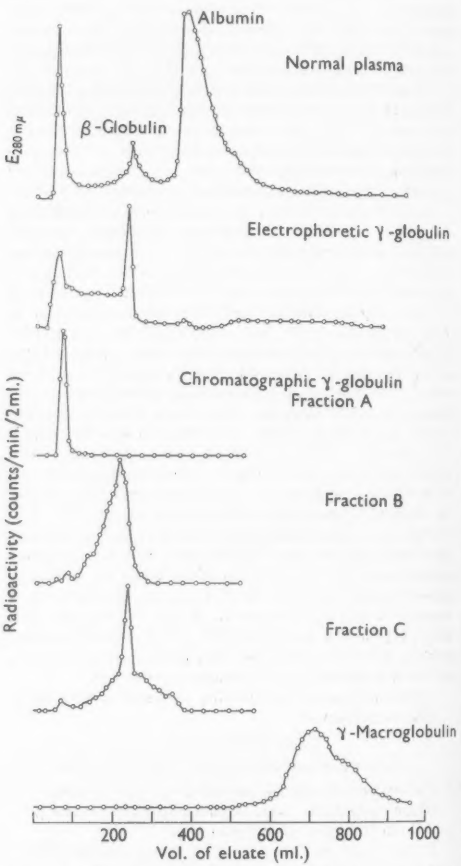

Fig. 5. Chromatographic analysis and radioactive assay of labelled γ-globulin preparations added to normal human serum. Protein distribution in normal serum is shown above. Less than 1 mg. of labelled γ-globulin was mixed with 10 ml. of human serum and analysed on 20 g. cellulose columns. The buffer was 5 mM-phosphate, pH 7·1, except with the electrophoretic fraction, in which 0·01 M-phosphate, pH 8·3, was used; the gradient was to 0·3 M-NaH_2PO_4. Radioactivity was measured by scintillation counting on volumes (2 ml.) of the eluate.

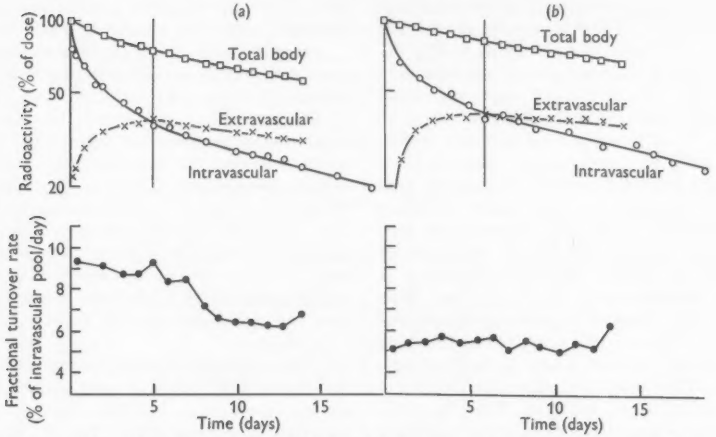

Fig. 6. Turnover data obtained with ^{131}I-labelled γ-globulin prepared by zone electrophoresis (*a*) and by chromatography (*b*). The equilibrium time is shown by a vertical line (see text).

intra-vascular γ-globulin, estimated by extrapolation of the linear portion of the plasma specific activity curve, was 1·2–1·6 (mean 1·4). Extravascular activity increased progressively to reach a maximum value after 3–5 days and thereafter declined more slowly than the intravascular activity.

Between 5·6 and 7·9 % of the injected ^{131}I was excreted in the urine within 24 hr. (Table 1). In the three subjects injected with γ-globulin prepared by sodium sulphate precipitation and electrophoresis, the fractional turnover rate calculated from the urinary excretion of ^{131}I declined progressively for 11–13 days (Fig. 7); strict control of urine collection was not possible in these subjects. The experiment was therefore repeated in two hospitalized subjects in whom urine collection was supervised (Expts. A 10, A 11, in Table 1). γ-Globulin prepared by zone electrophoresis was used; in both experiments the fractional turnover rate fell progressively for 7 days and then remained constant during a further week of observation (Fig. 7).

γ-Globulin prepared by chromatography. The three subfractions of γ-globulin prepared by chromatography and referred to as fractions A, B and C (Fig. 2) contained no rapidly sedimenting material when examined in the ultracentrifuge and each appeared as a single symmetrical peak; $S_{20,w}$ values were between 6·10 and 6·62 (Table 2). The hexosamine content of these fractions increased progressively from 1·18 to 1·62 % (Table 2); this finding could be correlated with increasing electrophoretic mobility (Fig. 5) and progressively later elution from the chromatographic column. Fraction A, when labelled and re-examined by chromatography, was confined to the first peak, fraction B emerged partly with β-globulin and fraction C extended to the albumin peak (Fig. 5).

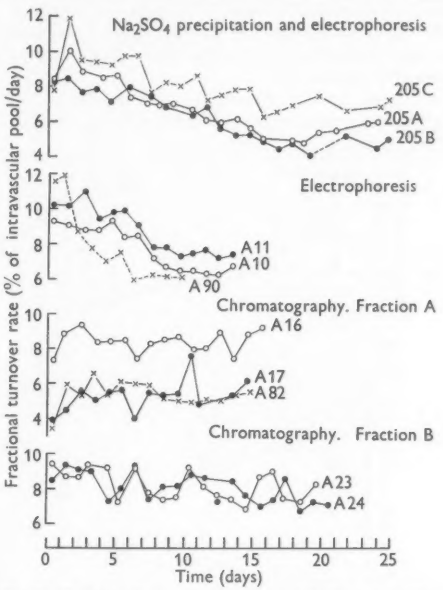

Fig. 7. Fractional turnover rate of γ-globulin preparations calculated from urinary excretion of ^{131}I. The broken line shows the fractional turnover rate obtained with a mixture of chromatographic fraction A and γ-macroglobulin (see text).

Five subjects were injected with either fraction A or B labelled with ^{131}I (Table 1); results obtained in an experiment with fraction A are shown in Fig. 6*b*. The turnover rate in all experiments was relatively constant throughout and did not show the initial progressive fall observed with electrophoretic γ-globulin (Fig. 7). The mean turnover rate varied from 5·2 to 8·4% in three subjects who received fraction A, and from 8·1 to 8·3% in two subjects injected with fraction B. Individual variation made it impossible to decide whether the two fractions were broken down at significantly different rates in healthy subjects. Fractions A and B were therefore separately labelled with either ^{131}I or ^{125}I, injected together and turnover rates compared directly in two subjects (Table 1 and Fig. 8, Expts. A 68 and A 80). In both experiments the two fractions were eliminated from the plasma at identical rates. In Expt. A 80 (Fig. 8) in which urine analyses were performed, the excretion of ^{131}I (fraction B) exceeded that of ^{125}I (fraction A) by 3·8% of the dose during the first 3 days of the experiment; thereafter, the excretion rates of the two isotopes were closely similar. The initial difference was probably due to the presence of denatured material in the ^{131}I-labelled protein as a similar result was observed in another subject given the same preparation. In Expt. A 85 (Fig. 8) fractions A and C prepared by salt precipitation and chromatography (Fig. 2*b*) gave identical plasma elimination curves and fractional turnover rates throughout.

Different methods of analysis gave similar values for the fractional turnover rate and distribution ratio of chromatographic γ-globulin (Table 3). The turnover rate was relatively high in the three convalescent patients (A 16, A 23 and A 24). In the remaining eight experiments performed on five normal subjects, the half-life of γ-globulin was 21–26 days (mean 23 days). The mass ratio of extra- to intra-vascular γ-globulin was 0·6–1·1 (mean 1·0) and the exchange rate between intra- and extra-vascular pools was equivalent to 25% (range 19–33%) of the circulating γ-globulin per day. Estimates of the absolute breakdown rate calculated from the slope of the plasma specific activity curve were always about 10% higher than values obtained by three other methods; the latter gave an absolute rate of 1·5–2·5 g./day (mean 2·1 g./day).

γ-Macroglobulin. The γ-macroglobulin prepared by ultracentrifuging and zone electrophoresis had an electrophoretic mobility similar to that of fraction B described above (Fig. 4). On chromatographic analysis, however, the macroglobulin was widely separated from γ-fractions of lower molecular weight and was eluted in the region beyond the albumin peak (Fig. 5).

Two subjects were injected with ^{131}I-labelled γ-macroglobulin (Table 1, A 53 and A 90). The distribution and turnover of this protein was strikingly different from other γ-globulin fractions studied (Fig. 9). The total radioactivity and intravascular radioactivity declined at similar rates and the slightly higher level of the former during the first 3 days can probably be attributed to the diffusion into the extravascular fluid of free iodine liberated during protein breakdown. This data

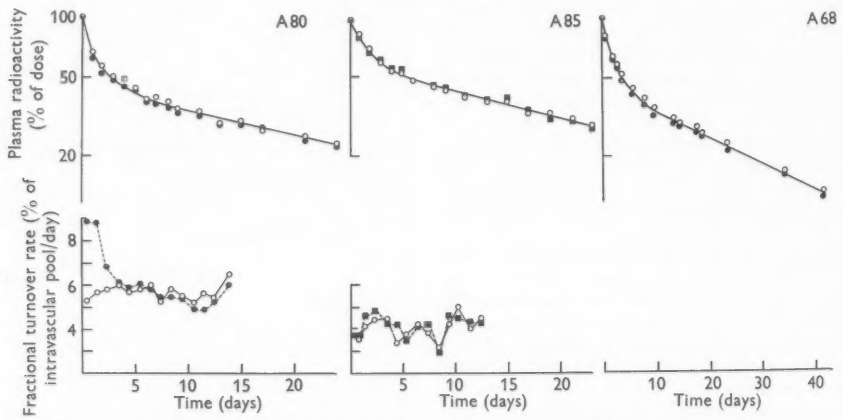

Fig. 8. Results in three subjects each injected with two different γ-globulin fractions prepared by chromatography. A 68, ^{131}I-labelled fraction A and ^{125}I-labelled fraction B; A 80, ^{125}I-labelled fraction A and ^{131}I-labelled fraction B; A 85, ^{125}I-labelled fraction A and ^{131}I-labelled fraction C. ○, Fraction A; ●, fraction B; ■, fraction C.

Table 3. *Turnover data in eight subjects injected with chromatographic subfractions of γ-globulin*

Intravascular γ-globulin mass = total intravascular protein × % of γ-globulin determined by paper electrophoresis. Distribution ratio (extra- to intravascular protein mass) was determined by (1) extrapolation of the linear portion of the plasma activity curve, (2) equilibrium-time method, (3) mathematical analysis (see text). Fractional turnover rate (FTR) was calculated from (1) half-life of plasma activity curve (% of total pool), (2) equilibrium-time method, (3) mathematical analysis, (4) urinary excretion of labelled iodine (mean value). Absolute turnover rate was determined from: (1) total exchangeable γ-globulin × FTR (method 1); (2) intravascular γ-globulin × FTR (mean of methods 2, 3 and 4). Exchange rate = % of intravascular γ-globulin passing into extravascular pool/day.

Expt. no.	Intravascular protein: Total (g.)	Intravascular protein: γ-Globulin (g.)	Fraction	Plasma vol. (l.)	Half-life (days)	Distribution ratio: 1	Distribution ratio: 2	Distribution ratio: 3	Fractional turnover rate/day: 1	Fractional turnover rate/day: 2	Fractional turnover rate/day: 3	Fractional turnover rate/day: 4	Absolute rate: 1	Absolute rate: 2	Exchange rate (%/day)
A16	195	27	A	2·58	14	1·2	0·9	0·7	5·0	9·1	9·8	8·4	3·0	2·5	28
A17	242	33	A	2·96	21	1·2	1·1	0·7	3·3	6·0	6·8	5·2	2·4	2·0	24
A82	186	28	A	2·48	26	1·1	0·9	0·8	2·7	5·3	5·5	5·4	1·6	1·5	33
A23*	156	24	B	2·34	—	—	1·1	1·1	—	7·5	9·5	8·3	—	2·0	30
A24*	197	29	B	2·78	—	—	1·3	1·4	—	8·4	8·6	8·1	—	2·4	28
A68	293	44	A	3·44	21	1·0	—	0·8	3·3	5·3	5·7	—	2·9	2·4	22
			B	3·40	21	1·2	—	0·8	3·3	5·6	5·7	—	3·2	2·5	22
A80	290	41	A	3·81	25	1·2	1·0	0·9	2·8	5·2	5·8	5·7	2·5	2·3	31
			B	3·80	25	1·2	1·0	0·9	2·8	5·5	5·8	6·1	2·5	2·4	31
A85	262	38	A	3·62	24	0·9	0·8	0·6	2·9	4·5	5·4	4·0	2·1	1·8	19
			C	3·65	24	0·8	0·9	0·6	2·9	4·0	5·4	4·1	2·0	1·7	19
Mean (excluding convalescent patients A16, A23, A24)				—	23	1·1	1·0	0·8	3·0	5·2	5·8	5·1	2·4	2·1	25

* In these subjects plasma specific activity did not decline at an exponential rate and the half-life could not therefore be determined.

Table 4. *Theoretical elimination rate of a minor component in electrophoretic γ-globulin*

For experimental details see text.

Expt. no.	Day	Observed excretion/24 hr. (% of dose)	Major component excretion/24 hr. (% of dose)	Minor component excretion/24 hr. (% of dose)	Minor component excretion/24 hr. (% of 9% of dose)	Minor component retained (%)
A10	1	7·3	5·2	2·1	23	77
	2	5·5	5·0	1·5	17	60
	3	4·7	3·6	1·1	12	48
	4	3·9	2·9	1·0	11	37
	5	3·6	2·7	0·9	10	27
	6	3·2	2·5	0·7	8	19
A11	1	7·9	5·8	2·1	23	77
	2	5·9	4·3	1·6	18	59
	3	5·2	3·4	1·8	20	39
	4	4·0	3·1	0·9	10	29
	5	3·8	2·8	1·0	11	18
	6	3·3	2·7	0·6	7	11

indicates that intact γ-macroglobulin does not penetrate into extravascular tissues, but remains confined to the circulation. The fractional turnover rate calculated from urinary ^{131}I fell progressively from an initial level of 35–38 % of the intravascular pool per day to 15–18 % after 5–7 days.

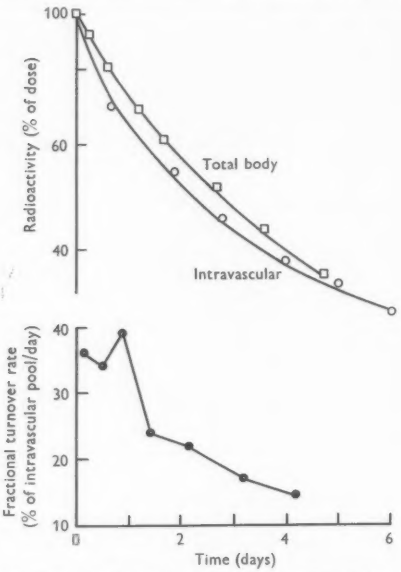

Fig. 9. Total body and intravascular radioactivity and fractional turnover rate in a subject (A 53) injected with ^{131}I-labelled γ-macroglobulin.

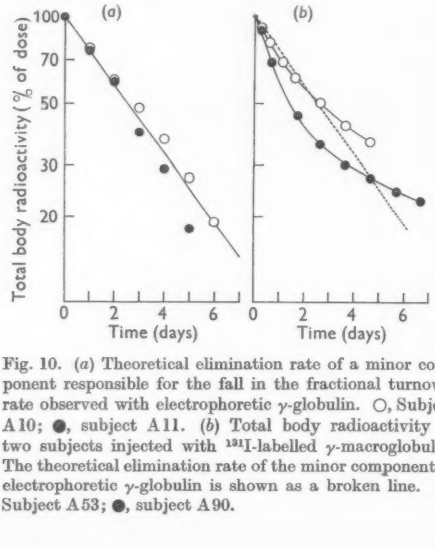

Fig. 10. (*a*) Theoretical elimination rate of a minor component responsible for the fall in the fractional turnover rate observed with electrophoretic γ-globulin. ○, Subject A 10; ●, subject A 11. (*b*) Total body radioactivity in two subjects injected with ^{131}I-labelled γ-macroglobulin. The theoretical elimination rate of the minor component of electrophoretic γ-globulin is shown as a broken line. ○, Subject A 53; ●, subject A 90.

An attempt has been made to calculate whether the initial fall in the fractional turnover rate observed with electrophoretic γ-globulin can be ascribed to the small amounts of γ-macroglobulin known to be present in these preparations. Chromatographic analysis of ^{131}I-labelled γ-globulin, prepared by electrophoresis, has shown that 9 % of the isotope is eluted as a broad peak in the same region as γ-macroglobulin (Fig. 5). From the data of experiments A 10 and A 11 it has been assumed that 91 % of the labelled protein has a constant turnover rate corresponding to the mean value observed during the second week of the experiment (6·6 %/day in A 10 and 7·5 %/day in A 11, Fig. 7). The daily excretion of ^{131}I resulting from the breakdown of this major component was calculated from $E' = P$/fractional turnover rate where E' is the daily excretion of ^{131}I from the major component (% of dose) and P is the mean level of intravascular radioactivity (% of dose) and the fractional turnover rate is that of the major component (% intravascular pool/day). The difference between E' and the observed daily excretion represents the radioactivity derived from breakdown of the minor component. This was expressed as a fraction of 9 % of the administered dose to obtain the theoretical elimination rate of the minor component (Table 4). This rate was similar for both experiments (Fig. 10*a*) and approximated to the observed elimination rate of γ-macroglobulin (Fig. 10*b*).

In one subject (A 90) ^{131}I-labelled γ-macroglobulin was injected together with chromatographic ^{125}I-labelled fraction A. The latter was eliminated at a constant rate equivalent to 6 % of the intravascular pool per day. The fractional turnover rate was calculated for a mixture of the two proteins assuming that 9 % of the total radioactivity was associated with macroglobulin. The result is plotted as a broken line in Fig. 7. The progressive fall in the fractional turnover rate during the first 6 days, characteristic of electrophoretic γ-globulin, was simulated by the mixture, but the rate of decline of the latter was more rapid.

DISCUSSION

A basic assumption in this investigation is that labelled γ-globulin preparations were not adversely affected by preliminary fractionation and labelling and were therefore metabolized in the same way as the corresponding native proteins. Electrophoresis and ion-exchange chromatography can be regarded as comparatively mild forms of protein fractionation. The turnover of rat albumin prepared by chromatography is unaffected by preliminary biological screening which indicates that the

original labelled material is virtually free of denatured protein (Cohen, 1958). Liver-perfusion experiments have shown that albumin, prepared either by chromatography or electrophoresis, contains less than 3% of molecules which have a relatively rapid rate of breakdown (Cohen & Gordon, 1958).

The validity of metabolic data obtained with ^{131}I-labelled proteins has been questioned in recent years (Goldsworthy & Volwiler, 1957). Studies in this Laboratory, however, have shown in animals that the behaviour of ^{131}I-labelled albumin and globulin is almost identical with that of the same proteins biosynthetically labelled with ^{14}C (Cohen *et al.* 1956; Campbell *et al.* 1956). Moreover, the elimination of ^{14}C- and ^{131}I-labelled antibody globulins is identical either in the presence or absence of an immune response (McFarlane, 1957; Humphrey, 1957). More recently, it has been shown that a subject with congenital analbuminaemia eliminated ^{131}I-labelled and unlabelled albumin at the same rate (Freeman, Matthews, McFarlane, Bennhold & Kallee, 1959; Bennhold & Kallee, 1959).

These experiments show that the radioactive iodine, attached under defined conditions, can be used as a reliable indicator of the metabolic behaviour of unlabelled protein molecules. In this connexion it may be noted that half-lives in this study fall within the range observed with biosynthetically labelled γ-globulin (Volwiler *et al.* 1955). The initial fall in the fractional turnover rate of electrophoretic γ-globulin therefore indicates that this preparation contains molecules which, in the native state, would have different rates of breakdown. This labelled γ-globulin had a wide distribution on chromatographic analysis corresponding with that described for unlabelled human γ-globulin (Fahey, McCoy & Goulian, 1958); the chromatographic similarity of ^{131}-labelled and unlabelled human albumin has been demonstrated (Cohen, 1959).

Turnover rates were constant with three chromatographic subfractions of γ-globulin and each must be regarded as metabolically homogeneous. The fact that a constant fraction of the plasma radioactivity was excreted each day, even during the process of equilibration between intra- and extra-vascular pools, indicates that γ-globulin breakdown occurs at a site in close functional proximity to the bloodstream; the same conclusion has been reached about the site of albumin catabolism (Berson & Yalow, 1954; Campbell *et al.* 1956; Lewallen, Berman & Rall, 1959). With a double-labelling technique the three chromatographic fractions were shown to have identical plasma-elimination and turnover rates. These fractions contained 10% of the serum protein applied to chromatographic columns from which the total recovery of protein was about 75%; taken together the fractions cover 90% of the electrophoretic γ-globulin chromatogram (Fig. 5). The results therefore indicate that the bulk of human γ-globulin has a uniform distribution and breakdown rate despite the fact that it can be resolved by chromatography into subfractions which have different electrophoretic mobility, total hexose (Fahey & Horbett, 1959) and hexosamine content, and distinct immunological activity (Humphrey & Porter, 1957; Sober & Peterson, 1958; Fahey & Horbett, 1959). This conclusion concerning the metabolic homogeneity of the bulk of human γ-globulin is not in accordance with the observation that individual antibodies are degraded at significantly different rates in hypo-γ-globulinaemic patients injected with pooled human γ-globulin (Martin, Gordon, Felts & McCullough, 1957).

The behaviour of the three chromatographic fractions does not explain the metabolic heterogeneity of electrophoretic γ-globulin. None of the chromatographic fractions contained macroglobulin, which was found to be eluted late in the chromatogram; other macroglobulin preparations have shown similar chromatographic behaviour on diethylaminoethylcellulose (Lospalluto & Ziff, 1959; Fahey & Horbett, 1959). Interpretation of the γ-macroglobulin turnover data must be qualified by the fact that the protein may have been altered during the repeated process of preparative ultracentrifuging despite the precautions taken to prevent denaturation. Thus the declining fractional turnover rate does not necessarily indicate that native macroglobulin would show similar metabolic heterogeneity. The fact that the elimination was initially somewhat faster than the theoretical rate (Fig. 10*b*) may be due to partial denaturation of the macroglobulin; similarly, the fractional turnover rate during the first 6 days fell more rapidly with a mixture of chromatographic and macroglobulin than with electrophoretic γ-globulin (Fig. 7). In most forms of denaturation altered protein molecules are very rapidly removed from the circulation (Freeman, 1959). The fact that the breakdown rate in two subjects was equivalent to 15–18% of the intravascular pool per day after 5–7 days indicates that the macroglobulin in its native form has a turnover rate at least three times as great as γ-globulin of smaller molecular size. A similar difference in the half-lives of small- and large-molecular-weight antibodies has been reported in rabbits (Taliaferro & Talmage, 1956).

The macroglobulin also differed from smaller-molecular-weight γ-globulin in its failure to penetrate into extravascular tissues. This apparent inability to pass across capillaries can be explained

in terms of the pore theory of permeability (Pappenheimer, 1953), but it is not easily accounted for by a more recent theory in which the transfer of droplets of plasma across the endothelium is postulated (Palade, 1956).

Methods of analysing turnover data rest upon the assumption that rates of synthesis, breakdown and exchange remain constant during the experiment. Analysis of the data obtained with a heterogeneous protein in terms of constant rates gives rise to considerable error; thus, for example, the conventional method of obtaining the pool ratio by extrapolation (Sterling, 1950) gives a value for electrophoretic γ-globulin of 1·22–1·66, whereas the same ratio for homogeneous protein fractions is 0·8–1·2. Calculations based upon the slope of the plasma specific activity curve are also dependent upon the assumption that specific activities are equal throughout the exchangeable protein pool after the period of equilibration. However, if protein breakdown is largely confined to the intravascular pool this assumption is not permissible (McFarlane, 1957); the extent of the resultant error is a function of the rates of turnover and exchange between compartments (Matthews, 1958). With human γ-globulin these rates are such that the error, as predicted by Matthews (1958), is small and the conventional analysis (Sterling, 1950) gives results comparable with those obtained by three other methods.

SUMMARY

1. ^{131}I-Labelled γ-globulin prepared by zone electrophoresis is metabolically heterogeneous; the fractional turnover rate falls progressively in healthy human subjects during the first 7–13 days after injection. Such γ-globulin preparations contain macroglobulin ($S_{20,w}$ 19) and after the labelling about 9% of the ^{131}I is attached to this fraction.

2. Three subfractions of γ-globulin of increasing electrophoretic mobility and hexosamine content, but having similar sedimentation coefficients ($S_{20,w}$ 6·10–6·62), were prepared by anion-exchange chromatography. These fractions, which comprised about 90% of the total γ-globulin, were metabolically homogeneous and all were identical in distribution and turnover in healthy subjects.

3. Four different analytical methods gave similar turnover data for the chromatographic γ-globulin fractions. The half-life was 21–26 days (mean 23 days), the extra- to intra-vascular mass ratio of γ-globulin was 0·6–1·1 (mean 1·0) and the exchange rate between intra- and extra-vascular pools was equivalent to 19–33% (mean 25%) of the circulating γ-globulin/day. The fractional turnover rate (% of intravascular pool/day) in normal subjects was 4·0–6·8% (mean 5·4%) and the absolute turnover rate was 1·5–2·5 g./day (mean 2·1 g./day).

4. γ-Macroglobulin was chromatographically distinct from the bulk of γ-globulin; its turnover rate was more rapid and it did not equilibrate with an extravascular pool.

5. These findings indicate that γ-globulin prepared by electrophoresis consists of two metabolically distinct groups of molecules which differ in regard to turnover rate and distribution between extra- and intra-vascular pools.

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REFERENCES

Bennhold, H. & Kallee, E. (1959). *J. clin. Invest.* **38**, 863.
Berson, S. A. & Yalow, R. S. (1954). *J. clin. Invest.* **33**, 377.
Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. E. & McFarlane, A. S. (1956). *Int. J. appl. Radiat. Isotopes*, **1**, 66.
Charlwood, P. A. (1955). *Canad. J. Chem.* **33**, 1043.
Cohen, S. (1958). *S. Afr. J. med. Sci.* **23**, 245.
Cohen, S. (1959). *Nature, Lond.*, **183**, 393.
Cohen, S. & Gordon, A. H. (1958). *Biochem. J.* **70**, 544.
Cohen, S., Holloway, R. C., Matthews, C. M. E. & McFarlane, A. S. (1956). *Biochem. J.* **62**, 143.
Dixon, F. J., Talmage, D. W., Maurer, P. H. & Deichmiller, M. (1952). *J. exp. Med.* **96**, 313.
Fahey, J. L. & Horbett, A. P. (1959). *J. biol. Chem.* **234**, 2645.
Fahey, J. L., McCoy, P. F. & Goulian, M. (1958). *J. clin. Invest.* **37**, 272.
Freeman, T. (1959). *Clin. chim. Acta*, **4**, 788.
Freeman, T., Matthews, C. M. E., McFarlane, A. S., Bennhold, H. & Kallee, E. (1959). *Nature, Lond.*, **183**, 606.
Goldsworthy, P. D. & Volwiler, W. (1957). *Ann. N.Y. Acad. Sci.* **70**, 26.
Gornall, A. C., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Humphrey, J. H. (1957). *Lect. sci. Basis Med.* **7**, 240.
Humphrey, J. H. & Porter, R. R. (1957). *Lancet*, i, 196.
Jencks, W. P., Jetton, M. R. & Durrum, E. L. (1955). *Biochem. J.* **60**, 205.
Lewallen, C. G., Berman, M. & Rall, J. E. (1959). *J. clin. Invest.* **38**, 66.
Lospalluto, J. & Ziff, M. (1959). *J. exp. Med.* **110**, 169.
McFarlane, A. S. (1957). *Ann. N.Y. Acad. Sci.* **70**, 19.
McFarlane, A. S. (1958). *Nature, Lond.*, **182**, 53.
Martin, C. M., Gordon, R. S., Felts, M. D. & McCullough, N. B. (1957). *J. Lab. clin. Med.* **49**, 607.

Matthews, C. M. E. (1957). *Physiol. med. Biol.* **2**, 36.
Matthews, C. M. E. (1958). *Radioaktive Isotope in Klinik und Forschung*, p. 310. München and Berlin: Urban und Schwartzenberg.
Müller-Eberhard, H. J., Kunkel, H. G. & Franklin, E. C. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 146.
Myant, N. B. (1952). *Clin. Sci.* **11**, 191.
Palade, G. E. (1956). *J. biophys. biochem. Cytol.* **2**, Suppl. 85.
Pappenheimer, J. R. (1953). *Physiol. Rev.* **33**, 387.
Perkins, W. J. & Piper, E. A. (1959). *Proc. int. Conf. Med. Electronics, Paris* (in the Press).
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Rondle, J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Sterling, K. (1950). *J. clin. Invest.* **30**, 1228.
Taliaferro, W. H. & Talmage, D. W. (1956). *J. infect. Dis.* **99**, 21.
Volwiler, W., Goldsworthy, P. D., MacMartin, M. D., Wood, P. A., MacKay, I. R. & Fremont-Smith, K. (1955). *J. clin. Invest.* **34**, 1126.

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The Lamellibranch Crystalline Style

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The crystalline style in many lamellibranchs exists as an elongated rod in a diverticulum of the stomach, the anterior end jutting into the stomach. It is rotated by cilia of the style sac, so that at the higher pH of the stomach, and by friction against the gastric shield, the one end frays and disperses, entangling food particles. The structure is constantly renewed by the epithelial cells lining the sac (see Nelson, 1918), and in anaerobiosis may disappear and then reappear under favourable circumstances. The style occurs in a few, but not many, gastropods, e.g. in the slipper limpet, *Crepidula fornicata*, L., *Patella* (Mackintosh, 1925), *Melanoides tuberculata* Müller (Fish, 1955).

The function of the style has been considered by many workers, and it is now generally agreed that its purpose is digestive. Coupin (1900) found in the style of *Cardium* amylase activity, as did Mitra (1901) in *Anodonta*. The properties of this enzyme in the oyster were studied by Yonge (1926), who also reported the absence of catalase, protease and lipase from the style. Though it is quite certain that proteolytic activity is absent, a lipolytic enzyme was found by George (1952) in the style of *Crassostrea virginica* Gmelin, the ribbed mussel *Modiolus demissus* Dillwyn and of other species; also by Hozumi (1959) in that of the clam *Venerupis philippinarum* Adams and Reeve.

Lavine (1946), noticing that Visking casing became weakened by style extracts from certain clams including *Mya arenaria* L., suggested the presence of a cellulase, though pure cellulose fibre was not digested. Since then, cellulase has been reported in the styles of *Ostrea edulis* L. and *Mytilus* (Newell, 1953); in the lamellibranch *Caelatura*, the gastropod *Melanoides* (Fish, 1955) and in the wood-boring lamellibranch *Bankia indica nair* (Nair, 1955). In *Crassostrea virginica*, Dean (1958) noted that various coloured algae suspended in a drop of sea water containing solid crystalline style became non-motile and the limiting membrane was indistinguishable after 10–15 min. This effect, which he calls 'a new property,' might appear too rapid to signify a cellulase, and it may well be due to an action on cell-wall lipid. It is not certain that the cellulase is derived from the secretory apparatus of the style sac, for it could be a component of the symbiotic spirochaetes usually found in the style. Walker & Warren (1938) studied a rather similar spirochaete (*Cytophaga*) isolated from soil, which decomposed cellulose partly to carbon dioxide and partly to a non-reducing mucilage.

A reinvestigation of phenol-oxidase activity in the style, first demonstrated by Berkeley (1923), has shown that for *Mytilus* and *Modiolus* at least, the enzyme has the character of an *ortho*-phenolase (Johansson, 1945). Its role is obscure.

Coupin believed that the style consisted mainly of a mucous substance 'saturated' with digestive fluid, and Barrois (1889) considered the style matrix to consist of globulin with a trace of 'mucin' or 'chondrin'. Mackintosh (1925) came to a similar conclusion, but in reality the tests employed were not strictly specific for true globulin. There seems no doubt, however, that a mucoprotein is present. During an investigation of the tropomyosin A (paramyosin) component of the

adductor of the large lamellibranch *Pinna nobilis* L. it was thought worthwhile to isolate the style and reinvestigate its nature in the light of newer methods developed for the study of mucoproteins. A preliminary account has already been published (Bailey, 1958).

MATERIALS AND METHODS

Treatment of styles. After storage at −20° the styles were dispersed in a homogenizer by addition of water and solid sodium bicarbonate to bring the pH to 8. A small amount of insoluble material (fraction 1) was spun down, and to the supernatant liquid dilute acetic acid was added to maximum precipitation at pH 5. Material remaining in the supernatant was precipitated with 2 vol. of acetone, and both precipitates were combined and redispersed at pH 8. The precipitation procedure was once more repeated, but this time the pH 5 and acetone precipitates were kept separate and freeze-dried (fractions 2 and 3 respectively). It seems unlikely that these fractions were actually different, since a 'pH 5 precipitate' after redispersing could again be divided into soluble and insoluble fractions.

An extract of the whole style was also investigated by dispersing as described above, dialysing against water and, after removal of a small sediment, it was precipitated with acetone after bringing to pH 5 with acetic acid ('whole-style fraction'). If to such an extract trichloroacetic acid is added (final concentration 2%), very little precipitation occurs unless the mixture be heated. This slight precipitate was thought to represent the various enzymes discussed above, and was dried in acetone and ether for further investigation ('enzyme fraction'). The supernatant, expected to contain the mucoproteins of the style, was neutralized to pH 5, and precipitated and dried in acetone ('mucoprotein fraction').

Methods of analysis. The methods used for the estimation of hexosamine, hexose and methylpentose have followed the general recommendations of Winzler (1955) and are derived from the following authors: hexosamine, the Elson–Morgan method as modified by Rimington (1940); protein-bound hexose, the method of Lustig & Langer (1931) as used by Weimer & Moshin (1952); methylpentose, the method of Dische & Shettles (1948); hexuronic acid, the carbazole method of Dische (1947); sialic acid, the 'direct' Ehrlich reaction as employed by Gottschalk (1955). The chromatographic separation of carbohydrates on paper was carried out in the ethyl acetate–pyridine–water mixtures of Jermyn & Isherwood (1949), and the spots were developed with aniline phthalate or with hexosamine reagents (Partridge, 1948).

Acetyl groups were estimated by the method recommended by Gibbons (1959), except that mixing of sample and conc. sulphuric acid was carried out in a freezing mixture before insertion in the Markham still.

Amino acid analysis was performed according to the improved column procedure of Moore, Spackman & Stein (1958) and amide nitrogen by direct estimation (see Bailey & Rüegg, 1960). Tryptophan was determined by the micro-method of Spies & Chambers (1949), edestin being used as standard. Hexosamines were also investigated by the method of Gardell (1953), and we are grateful to Mr D. F. Elsden for this part of the analysis.

RESULTS

General properties of style material. The fresh weight of the style of a large *Pinna* is 0·2–0·3 g., of which 80% is water. As already mentioned, trichloroacetic acid produces only a small precipitate and heating at pH 7 likewise produces only a slight haze. The atypical protein reaction of most of the material suggests that it consists of mucoprotein. Fraction 1, the undispersed material, represented only 2·5% of the total nitrogen and was not investigated; fraction 2 (pH 5 precipitate) weighed 0·30 g. and fraction 3 (acetone precipitate) 0·23 g. The weight of material precipitating with trichloroacetic acid ('enzyme' precipitate) is about 7% of the non-dialysable substance of a whole-style extract.

Carbohydrate analyses. Tests for uronic acid and sialic acid were entirely negative. Paper chromatography of hydrolysates of fractions 2 and 3 (4 hr. at 100° in 3% nitric acid and 10 hr. at 100° in 0·5N-sulphuric acid) showed the presence of hexosamine, galactose and fucose. Quantitative analysis with appropriate standards (galactose for hexose, rhamnose for methylpentose) gave the values reported in Table 1. There appear to be approximately equal amounts of hexosamine and hexose, and half as much methylpentose. In the whole-style extract the carbohydrate moiety, converted into anhydro residues, amounts to 26·2% by weight of the style substance. By column chromatography the hexosamine in fraction 2 was found to consist of equal parts of glucosamine and galactosamine (49 and 51% respectively), existing originally in the *N*-acetylated form. The analyses do not indicate that the various fractions are fundamentally different in chemical composition.

Surprisingly, the trichloroacetic acid precipitate (enzyme fraction) still contains considerable hexosamine, which suggested that the true enzyme protein which may be expected to precipitate under these conditions is very small indeed (see also below).

The character and relative amounts of the carbohydrate components are so like those of the blood-group substances (especially A group, see Kabat, 1956) that Professor W. T. J. Morgan kindly consented to test fraction 2 for A, B, H and Le^a activity. Moreover, Springer, Rose & György (1954) have reported that whole extracts of oyster possess a fairly strong group A activity. The results of our tests, however, were entirely negative.

Amino acid analysis. The results in Table 2 are expressed in terms of protein proper and not of whole-style substance, i.e. the α-amino nitrogen corresponding to a given amino acid is converted into the anhydrous weight of the amino acid, and all values are then summated. The total gives the amount of 'protein' derived from the column, and

individual values can now be expressed as a percentage of the total. In turn, these values can also be expressed as residues/10^5 g. of protein. Even if the recovery of individual amino acids is not absolutely quantitative, the losses are likely to be spread over the whole and will not cause serious error.

There is nothing very unusual in the amino acid pattern of whole-style protein, though it may be noted that, like the blood-group substances, the amounts of aliphatic hydroxy amino acids and proline are unusually high; but, unlike the blood-group substances, where aromatic residues are absent, there occurs a high proportion of these groups, especially of tyrosine. Cystine, methionine and tryptophan are also present. Although the amide nitrogen is corrected for ammonia arising by decomposition of hexosamine under the conditions of the distillation, it leads to the inference that the mucoproteins of the style possess an alkaline isoelectric point, i.e. base + amide groups exceed total dicarboxylic acids by 11 groups/10^5 g. of protein. Judged by the pH of minimum solubility around pH 5, the true isoelectric point appears to be on the acid side, and the amide value is probably still too high.

As noted above, the material precipitating in 2% trichloroacetic acid (enzyme fraction) still contains hexosamine, and the general pattern of amino acids present is not unlike the original material, except that the proline content is even higher, namely 124 groups/10^5 g. of protein, as against a value of about 40 which might be expected for material representing a mixture of enzymes (Bailey, 1950). This fact suggests that the enzyme activities demonstrable in the style constitute a very small part of the whole-style substance. It seems likely that this 'enzyme fraction' precipitates with trichloroacetic acid because of its lower content of carbohydrate.

In a hydrolysis of whole-style substance, the amount of amino acid eluted from the columns,

Table 1. *Carbohydrates of crystalline style*

Results are expressed as g./100 g. of dry material. — Indicates that no measurements were made.

	N	Hexosamine	Hexose	Fucose
Whole style*	11·2	11·2	12·2	5·7
Fraction 2	12·0	12·4	11·5	6·5
Fraction 3	12·4	10·8	8·3	—
Enzyme fraction	—	6·2	—	—
Mucoprotein fraction	—	13·5	—	—

* Theoretical acetyl as percentage of free hexosamine: 24·0; found: 20·7%.

Table 2. *Amino acid analysis of crystalline style*

	Wt. of anhydro amino acid/100 g. of protein*		Residues/10^5 g. of protein		
	Whole style	Enzyme fraction	Whole style	Enzyme fraction	Bovine oestrous mucoid†
Aspartic acid	10·87	14·0	95	122	59
Threonine	8·91	8·85	81	88	224
Serine	5·29	5·1	61	59	130
Glutamic acid	10·51	11·0	82	86	76
Proline	8·47	12·0	87	124	185
Glycine	4·38	6·35	77	111	71
Alanine	3·20	3·5	45	49	97
Cystine	4·28	0·9	42	9	Nil
Valine	5·33	5·5	54	56	58
Methionine	1·90	1·8	14	14	Nil
Isoleucine	4·19	3·9	37	35	15
Leucine	5·09	4·6	45	41	39
Tyrosine	10·12	6·1	62	37	?
Phenylalanine	4·82	4·1	33	28	29
Lysine	5·24	6·2	41	49	23
Histidine	1·84	1·7	13	12	Nil
NH_3	(1·51)	—	108	—‡	—
Arginine	4·07	4·1	26	26	34
Tryptophan	2·22	—‡	12	—‡	Nil
	100·7	99·8			

* Excluding the carbohydrate portion: for method of calculation, see text.
† Calculated from Gibbons (1959).
‡ Not measured.

assuming near-quantitative recovery, is also a measure of the protein moiety of the style. The analysis of Table 2 indicated a minimal protein content of 67%. The carbohydrates of Table 1, on the other hand, converted into anhydro residues and added to the acetyl content, amount to 28·6%, indicating a protein content of 71·4%. Considering the errors likely to exist in the carbohydrate determinations, these figures are very satisfactory.

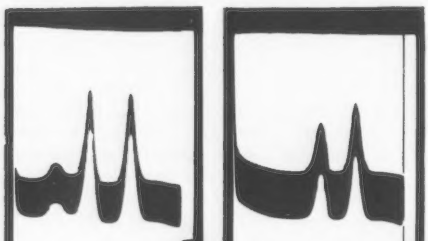

Fig. 1. Ultracentrifuge schlieren diagram for: (*a*) whole-style extract, concentration 1·5%; (*b*) a fractionated product (fraction 2), concentration 0·9%. KCl (*I* 0·1)–sodium phosphate (*I* 0·028), pH 7·5, was used. Exposure 64 min. after reaching full speed (269 000 *g* and 20°).

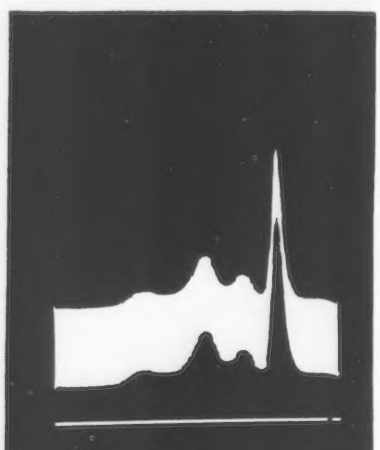

Fig. 2. Electrophoresis diagram of whole-style extract (ascending), concentration 1·5%. KCl–sodium phosphate was used as in Fig. 1; 100 v, 13 mA, 270 min.

Ultracentrifuge and electrophoretic data. In the ultracentrifuge the whole-style extract gives rise to three well-separated peaks (Fig. 1*a*); at a total concentration of 1·5% (pH 7·5, *I* 0·128) there appear a small fast component $S_{20,w}$ 12·4 and two major components $S_{20,w}$ 9·3 and 5·6. From the areas of the peaks the corresponding concentrations are 11, 47 and 42% respectively. In fraction 2 (Fig. 1*b*), the heaviest component has disappeared, and the peaks corresponding to $S_{20,w}$ 9·3 and 5·6 are present in concentrations of 42 and 58% respectively. Although no serious attempts at fractionation have yet been made, these results indicate that separation by simple methods is feasible.

With electrophoresis (Perkin–Elmer apparatus, model 38) the system is more heterogeneous, four distinct peaks being visible, in which the slowest is the largest and sharpest (Fig. 2). Taken together, the results of ultracentrifuging and electrophoresis suggest that the style mucosubstance is not so very heterodisperse as to make fractionation difficult: but it is possible, of course, that the discrete peaks seen in the ultracentrifuge pattern represent not one but a collection of molecules of somewhat differing size.

DISCUSSION

Gibbons (1959), in his detailed study of purified undegraded cervical mucin, has emphasized that in reality there are few sources of epithelial mucins from which pure, unmodified material may be obtained. They occur in the presence of proteolytic enzymes, or exist as a mixture of secretions of various types, or are physically modified by incubation at body temperature. The crystalline style is a good example of a secretion which is rapidly produced, easy to isolate and belongs entirely to one class of mucoid, that of the hexosamine–galactose–fucose mucoproteins. Though active enzymically, the actual amount of enzyme protein (amylase, lipase, cellulase) appears to be negligible in terms of weight.

In the ultracentrifuge, the style substance appears as a paucidisperse system, though one must be cautious in supposing that the three peaks each represent single components. The nature of this heterogeneity is unknown. Part of the style substance which precipitates with trichloroacetic acid has a lower hexosamine content than the original, suggesting that components of differing protein content exist; also the amino acid composition of this protein differs in certain respects from whole-style protein.

Little can be said about amino acid pattern other than that the proline–serine–threonine content is high, as in blood-group substances and cervical mucoids (see Table 2); but amino acids characteristically absent in these are present, with tyrosine in very large amounts.

SUMMARY

1. The nature of the mucoid comprising the substance of the lamellibranch crystalline style has been investigated in *Pinna nobilis* L.

2. It consists of approximately 30% of carbohydrate combined with 70% of protein. The carbohydrate portion is composed of *N*-acetylated glucosamine and galactosamine, galactose and fucose, which have been estimated. Although it resembles the blood-group substances in make-up, tests for A, B, H and Le^a activity were negative.

3. All the usual amino acids are present, with threonine–serine, proline and tyrosine in relatively large amounts.

4. By weight, the amount of enzyme protein (amylase, lipase, cellulase) is negligible, but the style mucoid itself, both electrophoretically and in the ultracentrifuge, behaves as a paucidisperse system.

REFERENCES

Bailey, K. (1950). *Chem. & Ind.* p. 243.
Bailey, K. (1958). *Biochem. J.* **69**, 45P.
Bailey, K. & Rüegg, J. C. (1960). *Biochim. biophys. Acta*, **38**, 239.
Barrois, T. (1889). *Rev. Biol. Nord du France*, **3–4**, 124, 161, 163; **5**, 209, 299, 351.
Berkeley, C. (1923). *J. exp. Zool.* **37**, 477.
Coupin, H. (1900). *C.R. Acad. Sci., Paris*, **130**, 1214.
Dean, P. (1958). *Science*, **128**, 837.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Fish, G. R. (1955). *Nature, Lond.*, **175**, 733.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
George, W. C. (1952). *Biol. Bull., Woods Hole*, **102**, 118.
Gibbons, R. A. (1959). *Biochem. J.* **73**, 209.
Gottschalk, A. (1955). *Biochem. J.* **61**, 298.
Hozumi, M. (1959). *Sci. Rep. Tokyo Bunrika Daig.* **9**, 37.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Johansson, J. (1945). *Ark. Zool.* **36**, no. 13, 1.
Kabat, E. A. (1956). *Blood Group Substances.* New York: Academic Press Inc.
Lavine, T. F. (1946). *J. cell. comp. Physiol.* **28**, 183.
Lustig, B. & Langer, A. (1931). *Biochem. Z.* **242**, 320.
Mackintosh, N. A. (1925). *Quart. J. micr. Sci.* **69**, 317.
Mitra, S. B. (1901). *Quart. J. micr. Sci.* **44**, 591.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Nair, N. B. (1955). *Curr. Sci.* **24**, 126.
Nelson, T. C. (1918). *J. Morph.* **31**, 53.
Newell, B. S. (1953). *J. Mar. biol. Ass. U.K.* **32**, 491.
Partridge, S. M. (1948). *Biochem. J.* **42**, 251.
Rimington, C. (1940). *Biochem. J.* **34**, 931.
Spies, J. R. & Chambers, D. C. (1949). *Analyt. Chem.* **21**, 1249.
Springer, G. F., Rose, C. S. & György, P. (1954). *J. Lab. clin. Med.* **43**, 532.
Walker, E. & Warren, F. L. (1938). *Biochem. J.* **32**, 31.
Weimer, H. E. & Moshin, J. R. (1952). *Amer. Rev. Tuberc.* **68**, 594.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
Yonge, C. M. (1926). *J. Mar. biol. Ass. U.K.* **14**, 295.

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Carbohydrate Metabolism in *Spirochaeta recurrentis*

1. THE METABOLISM OF SPIROCHAETES *IN VIVO* AND *IN VITRO**

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In contrast with the intensive studies on bacteria, very few investigations have been carried out on the biochemical activities of the order Spirochaetales, whose three main divisions comprise *Spirochaeta* (*Borrelia*), *Leptospira* and *Treponema*. The review by Geiman (1952) on spirochaete metabolism emphasizes how little is known of this interesting group of micro-organisms. The intermediate taxonomic position of spirochaetes between bacteria and Protozoa, and the difficulty in cultivating pathogenic types *in vitro*, may account for this neglect.

Earlier workers (Fenyvessy & Scheff, 1930; Scheff, 1935) reported that *Spirochaeta recurrentis*

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consumed glucose and produced lactic acid but did not use oxygen. McKee & Geiman (1950), using *Borrelia novyi* (another strain of relapsing-fever spirochaete), obtained similar results but indicated that there was a low consumption of oxygen. Comparable studies by Fulton & Spooner (1956) on *Leptospira icterohaemorrhagiae* showed the absence of a fermentative pattern of carbohydrate metabolism.

It was primarily because of the lack of any definite data upon the course of carbohydrate metabolism in spirochaetes that work on the biochemistry of *Spirochaeta recurrentis* was started. This paper serves as an introduction to further study.

METHODS AND MATERIALS

Parasite. The organism used for experiments both *in vivo* and *in vitro* was the tick-borne strain of *Spirochaeta recurrentis*, referred to in the literature by some authors either as *S. duttoni* or *Borrelia duttonii* (Breed, Murray & Smith, 1957), and was obtained from The Wellcome Laboratories.

Host. The strain was maintained throughout in albino rats weighing 70–80 g. Infected blood was obtained from two or three heavily infected donor rats by cardiac puncture under chloroform anaesthesia and mixed with 0·9% NaCl–2% (w/v) sodium citrate in the proportion of 1:1. A volume of 0·5–1 ml. of this mixture was inoculated intraperitoneally into other rats. The state of infection was judged daily by microscopical examination of tail blood. Peak infections of blood usually occurred 2 days after inoculation.

Experiments in vivo

Respiratory CO_2 in the rat host was measured in a metabolism cage based on that of McClintock & Lifson (1957), with modifications, so that samples of respiratory air could be taken at regular intervals and analysed for content of CO_2 by a simplified Haldane apparatus attached to the vessel in which the animal was housed. The vessel consisted of a desiccator, capacity 2 l., containing food bin, inlet and outlet for gases and one for drinking water. This arrangement avoided the tedious collection of large quantities of total CO_2 by the usual barium carbonate technique (Skipper, Bryan, White & Hutchinson, 1948).

Variations in the daily output of CO_2 by the rats under investigation were reduced to a minimum by taking samples between 10 a.m. and 4 p.m. each day, as recommended by Horst, Mendel & Benedict (1934). At the end of the first day the rat was removed from the apparatus, weighed and inoculated intraperitoneally with blood from another rat heavily infected with *S. recurrentis*. Analyses were then continued hourly throughout each day without removing the animal from the cage until the experiment was over. The rat was then taken out and weighed again.

Blood and liver samples from batches of normal and infected rats, for estimations of blood glucose, α-keto acids, lactic acid and liver glycogen, were always taken by cardiac puncture or excision of tissue immediately after killing by neck dislocation. Blood was run directly from the pipette into the protein precipitant to avoid variations caused by changes in the blood (Long, 1944), and liver samples were quickly weighed and plunged into the KOH solution used in the glycogen determination.

Experiments in vitro

Isolation of spirochaetes from blood. At the height of infection, when the blood was swarming with spirochaetes, the rats were anaesthetized by chloroform and bled by cardiac puncture into 0·9% NaCl–sodium citrate containing 0·2% of glucose. To this mixture was added 0·5 vol. of Krebs buffered medium (Krebs & Eggleston, 1940) at 37° containing 0·2% of glucose. After centrifuging at 250 *g* for 12 min. the cloudy supernatant was drawn off from the red blood cell residue and placed in 50 ml. plastic centrifuge tubes. The red blood cell layer was then washed twice more with the warm medium, centrifuged at 200 *g* for 10 min. and the supernatant also placed in plastic centrifuge tubes. The tubes were then centrifuged at 1500 *g* on an angle-head centrifuge for 4 min. exactly; the supernatant was discarded and a small amount of warm Krebs medium with glucose was added. On setting aside at a temperature of 30–35° the residue of motile spirochaetes begins to slide off the relatively tightly packed red and white blood cell layer and may be removed with a Pasteur pipette. This suspension was diluted further with warm 0·9% NaCl only and again centrifuged at 2000 *g* for 5 min. The thick layer of organisms was gently removed by agitating the 0·9% NaCl and the resultant suspension, which was essentially free of red and white blood cells, was used as such or diluted further with 5% (v/v) of rat serum in 0·9% NaCl containing 0·2% of glucose.

A curve correlating extinction, total nitrogen content (Kjeldahl) and dry weight (110°) was constructed. The density measurements were made on a Hilger Biochem Absorptiometer with a neutral-grey filter (Ilford H 503).

Manometric measurements. For measurement of either O_2 or CO_2 gas exchange use was made of the conventional Warburg constant-volume manometric apparatus (Dixon, 1943; Umbreit, Burris & Stauffer, 1949) and also, to a limited extent, the modified Scholander-type differential constant-pressure respirometer (Smith, 1958). In some experiments acid production was measured in the presence of bicarbonate buffer and N_2–CO_2 (95:5) atmosphere (Umbreit *et al.* 1949).

Chromatography. Paper partition chromatography was utilized for identification of metabolic end products produced *in vivo* and *in vitro*, the various modifications of the original method of Consden, Gordon & Martin (1944) being detailed below.

Identification of organic acids. Before chromatography, the suspension of organisms, previously incubated with glucose, was deproteinized with sodium tungstate (5%, w/v) and sulphuric acid (0·6N) and centrifuged. About 200 ml. of the deproteinized samples were neutralized with NaOH and concentrated *in vacuo* in a rotary evaporator. The solution was then acidified and distilled into dilute NaOH under slightly reduced pressure. The pH was adjusted to neutrality and the solution evaporated to near dryness. The residue of non-volatile material was also neutralized and evaporated to small volume.

The solution of salts of the volatile organic acids was acidified with an equal volume of orthophosphoric acid (60%, w/v) and the acids were obtained free from water by the method of James & Martin (1952*a*). The non-volatile acids were treated similarly. Solutions of the free acids were applied to paper and chromatographed with markers according to the procedure of Duncan & Porteous (1953) for volatile fatty acids, and that of Long, Quayle &

Stedman (1951) for the non-volatile acids. The zinc oxinate reagent of Pesez & Ferrero (1957) was also found useful in rendering the acids visible. The gas–liquid chromatography method of James & Martin (1952*a*, *b*) was also used in detecting volatile acids.

Identification of α-keto acids. Rats at the peak of infection were bled by cardiac puncture, heparin being used. After centrifuging the plasma was deproteinized and subjected to the Cavallini & Frontali (1954) procedure for the extraction of keto acids as 2:4-dinitrophenylhydrazones. These derivatives of pyruvic acid and α-oxoglutaric acid were used as markers and chromatographed by the technique of Altmann, Crook & Datta (1951).

Glycerol. For identification of glycerol, isolated by extraction of freeze-dried products by ethanol, chromatography was used as described by Grant & Fulton (1957).

Chemical methods

For reducing sugars the method of Nelson (1944) was used; for lactic acid that of Barker & Summerson (1941) as modified by Hullin & Noble (1953) was used, with lithium lactate as standard (Barker, 1957). Blood α-keto acid levels were measured by the method of Goodwin & Williams (1952), which was also used for estimating the production of pyruvic acid *in vitro* by the organisms.

^{14}C-Tracer studies. [Uniformly labelled (U)-^{14}C]- and [1-^{14}C]-glucose were used with suspensions of the live organisms and the distribution of radioactivity in the major products of metabolism *in vitro* was traced.

The final preparation of parasites from 25 rats was suspended in 15 ml. of Krebs buffered medium at 37°. Equal volumes (7 ml.) of the spirochaete suspension containing approx. 1·5 mg. dry wt./ml. were added to two volumetric flasks (50 ml.) each containing buffered medium + 0·3 % of glucose and about 0·75 μc of [U-^{14}C]- or [1-^{14}C]-glucose. In a third flask, which served as a control, an equivalent quantity of serum from which spirochaetes had been removed by centrifuging replaced the spirochaete suspension and [U-^{14}C]glucose was present in the flask. After making up to 50 ml. with buffered medium the entire contents of each flask were tipped into three × 250 ml. wide-mouthed conical flasks equipped with rubber-capped inlet and outlet tubes and a CO_2-absorption well containing pleated filter paper (Whatman no. 40). The flasks were kept at 35° and gas, either $N_2 + CO_2$ (95:5) or air + CO_2 (95:5) was passed through them via the tubes while they were shaken gently. At the end of the equilibration with the gas phase (10 min.) samples (1 ml.) were rapidly removed for initial glucose and lactate determination and also for glucosazone preparation and specific-activity measurement (10 ml.). The flasks were then stoppered tightly with the serum caps in place and shaken at 35°. At the end of 3 or 6 hr. N-NaOH (1 ml.) was added from a syringe with a thin needle, via the serum cap, to the inner tube containing filter paper and the contents of the flask were acidified with 2 N-H_2SO_4 (1 ml.) via the other capped tube. The flasks were shaken at an increased rate for 20–30 min. to aid absorption of the CO_2, and at the end of this time the absorption tubes were removed, the traces of alkali on the serum-capped tubes were rinsed into them with a few millilitres of CO_2-free water and then more CO_2-free water was used to elute $Na_2{}^{14}CO_3$ from the paper. The absorption tubes were rubber-stoppered and left standing for 12 hr. Samples of the incubation medium were withdrawn from the flasks for final glucose and lactate determination. The remainder of the flask contents was then used for preparation of lactate.

Preparation of glucosazone. General procedures for preparation of radioactive samples are described by Steinberg & Udenfriend (1957). The method for the preparation of glucosazone containing ^{14}C isotope was that of Feller, Strisower & Chaikoff (1950). A minimum of two recrystallizations of the glucosazone were generally carried out before constant specific radioactivity was achieved.

Preparation of $Ba^{14}CO_3$. The filter paper was removed from the CO_2-absorption tube and together with CO_2-free water was used to elute $Na_2{}^{14}CO_3$, placed in a flask and again washed with water. The supernatant was centrifuged in stoppered tubes to remove cellulose fibres and the $Ba^{14}CO_3$ precipitated by addition of $BaCl_2$ (12 %, w/v) containing a small amount of NH_4Cl. The precipitate was washed carefully with water, transferred quantitatively to a watch glass and dried.

Isolation of labelled lactate. A measured volume (30 ml.) of the incubation medium was removed from the test flasks and about 100 mg., accurately weighed, of pure anhydrous zinc lactate added to each. After deproteinization with sodium tungstate (5 %, w/v) and H_2SO_4 (0·6 N) the supernatant was continuously extracted with warm ether for 24 hr., and the lactic acid was isolated according to the method of Bernstein & Wood (1957). The acid was then neutralized with zinc carbonate, boiled to destroy the inner lactone and, after evaporating to the point of crystallization in a stream of air, ethanol–acetone (90:10) was added until a faint turbidity appeared. On setting aside at 0° for a few hours crystals of zinc lactate formed. These were collected and recrystallized from ethanol–acetone. The salt was then dehydrated in an air oven at 110° and stored in a vacuum desiccator.

Isolation of labelled glycerol. Extraction of glycerol from the incubation medium by deproteinization and evaporation to dryness over P_2O_5, followed by extraction with ethanol and chromatography, was carried out according to the method used by Grant & Fulton (1957). Ethanolic eluates from the chromatographic strips were pooled, concentrated to small bulk in a ground-glass stoppered tube, and the glycerol present was benzoylated by adding 0·5 ml. of 30 % (w/v) NaOH, followed by cautious addition of a few drops of benzoyl chloride with shaking. After the reaction was complete the solid was washed on the filter with a large volume of water and the residue dried in a desiccator over $CaCl_2$. In view of the small yield, no recrystallization was attempted.

Isolation of spirochaete material. After incubation the organisms were isolated by centrifuging, washed twice in 0·9 % NaCl and freeze-dried. The dry material obtained was used for counting without further treatment.

Specific activity of samples. Measurements of radioactivity were made on 0·3 cm.^{2} Perspex planchets that had been previously calibrated to obtain the factor for conversion into planchets of 1 cm.^{2} area. Counts of the amounts of radioactivity in the different samples, whether $BaCO_3$, glucosazone or zinc lactate (Karnovsky *et al.* 1955), were made on a Dynatron scaler with thin end-window Geiger–Müller counter, at infinite thickness, unless otherwise specified. In cases where samples were not infinitely thick correction factors were applied (Calvin, Heidelberger,

Reid, Tolbert & Yankwich, 1949). Correction of the lactic acid activity to allow for the added carrier was made by means of the equation given by Arnstein & Grant (1957). Where warranted, coincidence corrections for the particular counter used were made. The usual error associated with radioactive measurements of this kind arising out of variation in preparation of samples and statistical errors in counting was estimated to be ±5%.

Reagents. General reagents were of AnalaR grade. The purest obtainable specimens of sugars were supplied by British Drug Houses Ltd. [U-^{14}C]- and [1-^{14}C]-Glucose were purchased from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Metabolism of Spirochaeta recurrentis *in laboratory rats*

The suitability of a number of laboratory animals was tested for this strain of parasite normally maintained by serial passage in mice. Animals tested were the multimammate rat (*Mastomys coucha*), gerbils (*Meriones libicus*), hamsters (*Mesocricetus auratus*), guinea pigs and rats, and the most suitable was the albino rat, of weight 70–80 g. Twenty of these heavily infected animals provided sufficient material for experiment.

The disease in rats is rarely fatal, although their food and water consumption is reduced during infection. Leucocytosis is marked, the spleen and liver are enlarged and, when infections become heavy, bile pigments are present in the blood. The peak of infection is reached in 2 days with the inoculum used and falls to zero in a matter of hours without reappearance of parasites over a period of 6 weeks. During this time the rats cannot be reinfected. If the blood of animals from which the parasites had disappeared was added to spirochaetes, used for inoculation of fresh animals, marked clumping of the parasites took place and the resulting degree of parasitaemia was reduced.

Biochemical changes in the host during infection

Preliminary work on the chemical changes brought about in the rat by the spirochaete was undertaken to obtain information on the nature of the metabolism of the parasite. Blood-glucose concentrations were found to be decreased proportionally to the degree of parasitaemia as with other blood parasites (Christophers & Fulton, 1938; Mercado, 1952). Detailed estimation of blood glucose and liver glycogen revealed that there was a fall in glucose and an even greater fall in liver glycogen (see Table 1), in spite of the fact that experiment showed that the host food intake was not seriously reduced.

Examination of the end products of metabolism in the host blood

The large demands made by the spirochaetes on the blood glucose of the rat host led to a search for α-keto acids, but only pyruvate was detectable. Lactic acid was also found to be present in large amounts. The determination of these two substances in the blood of normal and infected animals was therefore undertaken.

The normal value of pyruvate in rat blood is about 0·7–1·1 mg./100 ml. (Goodwin & Williams, 1952). In our normal host animals the value was 1·6 mg./100 ml. and this value was trebled in rats infected with *S. recurrentis* (Table 1). The level of lactic acid in the host blood rose suddenly until at the height of infection its value was also trebled. The lactic acid values fell rapidly as the parasites disappeared from the blood. In view of the large amount of acidic products present in the blood during infection the respiratory CO_2 output was measured. From the results shown in Table 1 it is apparent that rats expire significantly more CO_2 only at the peak of infection. In view of the above findings the investigation *in vitro* described below was undertaken.

Metabolism of Spirochaeta recurrentis in vitro

S. recurrentis is a fragile organism and when isolated from formed blood elements it was found that motility could best be preserved when cells were suspended in buffered medium–glucose at 35° with gentle agitation. The addition of albino rat serum up to 5% was beneficial, possibly by reason of its buffering capacity. In medium alone, with no energy source, aggregation readily occurred. Similarly, clumping was brought about by horse or rabbit serum. A useful index to the vitality of spirochaetes was provided by motility observed

Table 1. *Biochemical changes in the rat during laboratory infection with* Spirochaeta recurrentis

Average values and variations of the mean are given.

Period of infection*	No. of rats	Av. wt. (g.)	Glucose (blood) (mg./100 ml.)	Glycogen (liver) (mg./100 ml.)	Liver wt. (g.)	Pyruvate (blood) (mg./100 ml.)	Lactate (blood) (mg./100 ml.)	Respiratory CO_2 (g./hr./100 g. rat)
Controls	23	85±5	100·4±2·2	4·96±0·44	3·8±0·3	1·60±0·07	47·6±2·3	0·456±0·085
1	10	86±4	—	—	—	—	75·6±5·9	0·496±0·047
2	23	76±5	43·7±3·5	1·51±0·30	4·7±0·2	4·58±0·06	146·0±10·6	0·771±0·037
3	10	77±3	—	—	—	—	61·0±3·8	0·472±0·034

* Figures indicate: 1, first day after infection; 2, at peak of infection; 3, day after peak of infection.

under dark-ground illumination. Since the parasites were observed to lose motility in the absence of glucose this method was used as a test for the specificity of various substrates (Fulton & Spooner, 1956).

Substrate specificity. The final suspension of organisms as isolated from the blood was diluted and washed two or three times with warm 0·9% NaCl until all were immobilized, care being taken not to keep them too long in the absence of sugar. The energy sources used and results obtained are given in Table 2.

It is clear that the chief substrates required by *S. recurrentis* are glucose and fructose. The changes in pH value indicate that with glucose an appreciable amount of acid is formed. Lactose, maltose and glycerol also appeared to support motility to a smaller extent. Neither galactose nor the pentoses were metabolized.

Table 2. *Substrate specificity for maintenance of motility in* Spirochaeta recurrentis

Flasks (in duplicate) contained substrate (5 mM, 10 mM), immobilized spirochaetes (0·1–0·2 mg./ml. dry wt.) and 0·9% NaCl to a total volume of 5 ml. Blanks were run with no substrate. pH at start, 7·4. Temp. 35°. In air. Duration of expts. 1 hr.

	Activity*		ΔpH	
Substrate	5 mM	10 mM	5 mM	10 mM
D(+)-Glucose	+++	+++	−0·8	−1·2
D(−)-Fructose	++	+++	−0·7	−0·8
D(+)-Mannose	−	−	−0·1	−0·1
L(−)-Sorbose	−	−	0·0	0·0
D(+)-Galactose	−	−	−0·1	0·0
-Lactose	+	+	−0·2	−0·2
-Maltose	+	+	−0·3	−0·3
D(+)-Ribose	−	−	−0·1	−0·1
D(+)-Arabinose	−	−	0·0	−0·1
-Glycerol	−	+	−0·2	−0·2

* Symbols indicate: −, no spirochaetes motile; +, a few spirochaetes alive but majority immobile; ++, half of total motile; +++, all vigorously motile.

Gaseous exchanges. Preliminary experiments with *S. recurrentis in vitro* failed to show an uptake of O_2 or CO_2 production except when white blood cells had not been completely removed from the preparation. There was, however, a marked utilization of glucose to the extent of a half to three-quarters of the dry weight of the organisms/hr.

The evolution of CO_2 mentioned by earlier writers (Fenyvessy & Scheff, 1930; Scheff, 1935) remained to be explained. Experiments were therefore designed to measure gas exchanges in *S. recurrentis* under varying experimental conditions, with concomitant estimation of glucose used and lactic acid produced. These results are shown in Table 3. There is no apparent O_2 uptake or CO_2 output by the organisms themselves. The stoicheiometric relations between glucose used and lactic acid formed show that the reactions were predominantly glycolytic in character.

Identification of end products of spirochaetal metabolism

Paper chromatography of the volatile and non-volatile organic acid fractions was carried out as described above. Only lactic acid was detected. By the more sensitive method of gas-phase chromatography, however, traces of *n*-butyric acid, *iso*valeric acid and *n*-valeric acid were found (Fig. 1). Pyruvic acid and traces of glycerol were also detected by the methods described. The presence of appropriate controls confirmed that these end products were the result of glucose catabolism.

In view of these findings an attempt was made to obtain a balance between glucose utilization and pyruvate and lactate production *in vitro* (Table 4). It appears that the utilization of glucose is accounted for almost completely by the lactic acid and pyruvic acid produced. The increase of pyruvate with time is thought to be due to accumulation of lactate causing reversal of the equilibrium

Table 3. *Origin of carbon dioxide in suspensions of* Spirochaeta recurrentis

Warburg flasks contained spirochaetes plus Krebs medium, or, in 30% (v/v) concentration, normal rat serum or rat serum which had been first acidified, evacuated and then neutralized, or whole rat blood. Energy source was glucose (0·3%). Blanks contained all the above-mentioned except spirochaetes. Centre wells of flasks measuring O_2 uptake contained 0·2 ml. of 20% KOH. Side arms of flasks measuring CO_2 output contained 0·5 ml. of 2N-H_2SO_4, which in some flasks was tipped before, and in others after, the experiment, to allow for CO_2 retention by the medium. Temp. 37°. Gas phase, air. Total liquid volume, 3·2 ml. Duration of expts. 1 hr.

	Total O_2 uptake (μl.)		Total CO_2 output (μl.)					
Suspending medium	Spirochaetes	Blank	Spirochaetes	Blank	Total glucose utilization (μmoles/ml.)	Total lactic acid production (μmoles/ml.)	Glucose:lactic acid ratio (molar)	Glucose converted into lactic acid (%)
Krebs medium	0	2	16	12	5·83	10·74	1:1·84	92
Serum	2	0	73	69	7·55	14·67	1:1·94	96
Treated serum	0	0	15	8	3·42	6·66	1:1·96	98
Whole blood	36	50	125	124	10·13	19·72	1:1·95	97

pyruvate⇌lactate in favour of pyruvate production. The predominantly glycolytic nature of the reactions taking place is again apparent.

Acid production as a measure of glycolytic activity and inhibition by various reagents. When the spirochaetes were suspended in 0·9% NaCl containing 0·2% of glucose in the presence of 0·01 M-sodium bicarbonate the CO_2 produced in the presence of different substrates could easily be measured. The results confirmed the substrate-specificity tests described in which glucose was found to be the primary energy source. The same conditions were used to measure inhibition of acid production, as described in Table 5. These conditions are closely similar to those used by Lardy & Phillips (1943*a*, *b*) and by Mann & Lutwak-Mann (1948) in their work on sperm glycolysis and respiration. The pH was approximately 7·0 when a gas phase of N_2+CO_2 (95:5) was used as 37° (Umbreit *et al.* 1957). The inhibition by fluoride is reversible, a phenomenon first noted by Lardy & Phillips (1943*b*) during studies on sperm glycolysis. Iodoacetate inhibition of acid production by spirochaetes was complete and irreversible. As would be expected in organisms without aerobic requirements the addition of azide to the suspension medium makes little or no difference to the metabolic rate. By far the most marked effect was obtained by pyruvate. This reagent is well known as a glycolytic stimulant in certain concentrations (Mendel, Bauch & Strelitz, 1931; Bumm, Appel & Couciero, 1933) and, in fact, at the smaller concentration used here a 50% stimulation of the rate of acid production occurred. The reactions were slowed when the pyruvate concentration was raised. The addition of adenosine triphosphate and Mg^{2+} ions failed to cause any enhancement of the rate of acid production, and likewise the addition of several sugar phosphate esters was without effect, owing possibly to permeability factors.

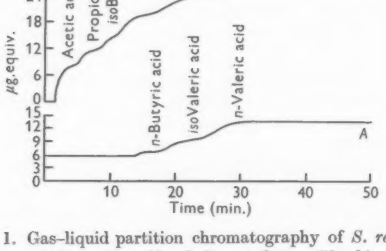

Fig. 1. Gas–liquid partition chromatography of *S. recurrentis* volatile acids. Metabolic products (50 ml.) were neutralized and freeze-dried. The dry residue was taken up in water (5 ml.) and an equivalent volume of orthophosphoric acid (60%, w/v) added. The mixture was placed on a Celite–Na_2SO_4 (anhyd.) column, eluted with dry ether, evaporated and distilled on to an acid-washed Celite 545 column. Column length, 4 ft.; liquid phase stearic acid (10%, w/v) in DC 550 silicone; N_2 pressure, 47 cm. Hg; temp. 100°. *A*, Test sample; *B*, marker sample.

Experiments with radioactive glucose

The results shown in Table 6 provide additional proof that CO_2 is not given out during the metabolism of *S. recurrentis in vitro*. The very small evolution of CO_2 recorded is insignificant, being of the order of 0·2% of the total glucose utilized when allowance is made for blank experiments. The picture does not appear to be markedly different with either [U-^{14}C]- or [1-^{14}C]-glucose.

Table 7 shows as well that no incorporation of radioactive label occurs in either the spirochaetes themselves or in the small amount of glycerol formed under the conditions of the experiment.

DISCUSSION

It has been shown in these studies that *S. recurrentis* consumes large amounts of glucose *in vitro*. Since the glucose content of the host blood is decreased in proportion to the degree of parasitaemia, a fact first noted by Fenyvessy & Scheff (1930), it is not unreasonable to suppose that this substrate plays an important part in the metabolism of the parasite *in vivo*. Support for this view is provided by the fact that these rats were allowed food without restriction and intake was not significantly reduced except for a few hours at

Table 4. *Glucose, lactate and pyruvate balance in* Spirochaeta recurrentis *metabolism* in vitro

Suspension (25 ml.) of *S. recurrentis* in medium + glucose (0·2%) was incubated at 35° on a shaker in air. Samples were withdrawn at zero time and at intervals during the course of incubation.

Time (min.)	Glucose utilized (μmoles/mg. dry wt.)	Lactic acid formed (μmoles/mg. dry wt.)	Pyruvic acid formed (μmoles/mg. dry wt.)	Lactic acid (% glucose converted)	Pyruvic acid (% glucose converted)
30	8·7	16·8	0·8	93·0	4·6
60	16·7	30·0	2·0	90·0	6·0
120	23·7	46·0	3·6	97·0	7·6
180	25·5	47·8	4·4	94·0	8·7

Table 5. *Inhibition and stimulation of* Spirochaeta recurrentis *metabolism*

Warburg flasks contained 1 ml. each of spirochaete suspension, glucose (24 μmoles) and 0·01 M-sodium bicarbonate in 0·9% NaCl. Side arms contained 0·5 ml. of solution of inhibitor or stimulator to give the indicated molarity. Duplicate samples of suspension, treated with 0·1 M-potassium fluoride and incubated for 30 min., were washed three times with 0·2% glucose–0·9% NaCl to remove fluoride and set up similarly to controls. After equilibration flasks were tipped. Temp. 37°. Gas phase N_2+CO_2 (95:5). Total vol. 3·5 ml.

Additions	Concn. (M)	CO_2 (μl./hr./mg. dry wt.)	Inhibition (−) or activation (+) (%)
Fluoride	0·10	5±3	97 (−)
Azide	0·001	165±15	8·8 (+)
Iodoacetate	0·0001	0±3	100 (−)
Pyruvate	0·10	38±5	75 (−)
	0·001	234±23	55 (+)
Control	—	151±14	0
Washed	—	142±8	6·6 (−)

the peak of infection. In malaria and trypanosome infections the liver-glycogen content is also decreased (Christophers & Fulton, 1938; Mercado & Brand, 1954). There is a marked increase in lactic acid and pyruvic acid in the blood, in agreement with the production of these substances *in vitro* by the parasite. Coleman & Brand (1957) have shown that during haemoprotozoan infections in the rat pyruvic acid concentrations in the blood are raised. The pronounced rise in the blood concentrations of lactic acid in spirochaete-infected rats doubtless throws some strain on the buffering capacity of the host blood and disposal of lactic acid as carbon dioxide and water seemed probable. We have confirmed this experimentally with carbon dioxide. The study of gaseous exchanges in trypanosome-infected rats by Brand (1951) has shown that a greater amount of carbon dioxide is given out by infected animals in the 36 hr. preceding death, when parasites become very numerous.

It seems strange that in an aerobic environment this parasite should not consume any oxygen. This finding is in agreement with that of Fenyvessy & Scheff (1930) later confirmed by Scheff (1935). McKee & Geiman (1950), using a North American strain of relapsing-fever spirochaete, reported that there was a small oxygen uptake by the parasite *in vitro*, but actual figures were not given. It is well known (Hindle, 1931) that a pronounced leucocytosis always occurs when the parasites are at the peak of infection. As leucocytes are known to have a high rate of aerobic respiration (Coxon & Robinson, 1956) it is possible that this could explain the oxygen consumption noted by these authors.

Results obtained in the experiments described in Table 3 show clearly that there is no carbon dioxide production by the organisms themselves. The observation by earlier workers that carbon dioxide was produced by the organisms apparently was based on the fact that addition of fresh normal rat serum to the suspension caused increased gas production, adequate controls were lacking. From the stoicheiometric relations given in Tables 3 and 4 it seems that the metabolic reactions of the spirochaetes are predominantly glycolytic in character. This requires a 1:2 molar relationship between glucose utilized and lactic acid produced, which in fact was found to be the case. The presence of a glycolytic pathway (or a minor variant or both; Entner & Doudoroff, 1952) is suggested by the results obtained with an inhibitor such as fluoride, which interferes with a phosphorylative type of metabolism (Claridge & Werkman, 1953). Further, the effect of iodoacetic acid in small concentration on enzymes containing essential sulphydryl groups (Barron, 1951) selectively inhibits triose phosphate dehydrogenase (Yang, 1957).

In contrast with the relatively large amounts of lactic acid produced by *S. recurrentis*, *n*-butyric acid, valeric acid and *iso*valeric acid occurred only in amounts detectable by delicate techniques of gas-phase chromatography and may in the absence of acetic acid be of significance only in the amino acid or fat metabolism of the organism as coenzyme A derivatives (Rendina & Coon, 1957; Masoro, Felts, Panagos & Rapport, 1957).

The presence of a higher amount of radioactivity in the evolved carbon dioxide from breakdown of [1-^{14}C]glucose, compared with that from the [U-^{14}C]- or [6-^{14}C]-species, is taken to indicate, with certain reservations, the operation of a shunt mechanism of the hexose monophosphate type (Wood, 1955; de Duve & Hers, 1957). This method of determining metabolic activity has been used by various workers (Bloom, Stetten & Stetten, 1953; Bloom & Stetten, 1953; Santer & Ajl, 1955; and Coxon & Robinson, 1956). The assumption made is that the carbon in position 1 of the 6-phosphogluconate molecule is split off as carbon dioxide under the action of 6-phosphogluconate dehydrogenase if the hexose monophosphate pathway is operative. Consequently, if all the radioactivity is centred at the C_1 site in the glucose any carbon dioxide liberated will contain all the label from this source. Further the recent assessment of the isotopic method for evaluation of the routes of glucose utilization in micro-organisms by Dawes & Holms (1958) emphasizes the weighting of the evidence in favour of the presence of a hexose monophosphate pathway by use of [1-^{14}C]glucose. Therefore the finding that in *S. recurrentis* no $^{14}CO_2$ is evolved, even with glucose labelled at C_1, is evidence strongly in favour of the non-existence of an oxidative cycle of carbohydrate metabolism.

Table 6. *Distribution of radioactivity from labelled glucose* in vitro: *distribution of* ^{14}C *among metabolic products*

Spirochaete suspensions (50 ml.; 100 ml. in the preliminary experiment) containing [U-^{14}C]glucose (0·3%; approx. 0·75 μc) or [1-^{14}C]glucose (0·3%, approx. 0·75 μc) were incubated at 35° under the conditions and for the times indicated. Glucose and lactate were estimated colorimetrically at the beginning and end of each experiment, and were counted as glucosazone and anhydrous zinc salt respectively. Carbon dioxide was absorbed in NaOH and counted as $Ba^{14}CO_3$. —, Signifies value not determined. Controls contained the same constituents and activity as described above but no spirochaetes.

Experimental conditions and label used	Duration (hr.)	Specific radioactivity (μc/g.) Glucose	Lactic acid	Original activity (%)	Total radioactivity utilized or produced (counts/min.) Glucose	CO_2	Total activity (%)
Preliminary air; [U-^{14}C]glucose + Krebs medium alone	6	4·34	—	—	26 700	62	0·23
Aerobic; [U-^{14}C]glucose	3	2·07	2·08	>100	7 740	31	0·40
+5% serum [1-^{14}C]glucose	3	1·97	1·96	99	7 630	38	0·49
Control; [U-^{14}C]glucose	3	—	—	—	—	22	—
Anaerobic; [U-^{14}C]glucose	3	2·04	1·94	95	6 925	25	0·36
+5% serum [1-^{14}C]glucose	3	2·27	2·28	>100	8 200	52	0·63
Control; [U-^{14}C]glucose	3	—	—	—	—	17	—

Table 7. *Distribution of radioactivity from labelled glucose* in vitro: *distribution of* ^{14}C *among organisms and glycerol*

Experimental details were as given in Table 6. Spirochaetes were isolated and glycerol was extracted after incubation as described in the Methods and Materials section.

Experimental conditions and labelled glucose used	Duration (hr.)	Specific activity (μc/g.) Glucose	$BaCO_3$	Spirochaetes	Glycerol
Anaerobic; [U-^{14}C]glucose + 5% serum	6	1·475	0·000	0·014	0·120
Control; [U-^{14}C]glucose	6	1·659	0·040	—	—

It must be concluded therefore that as the major portion of the ^{14}C label in glucose appears quantitatively in the lactic acid formed, without loss by way of carbon dioxide, anaerobic glycolysis by the Embden–Meyerhof route must be the only pathway of glucose catabolism present *in vitro* in *S. recurrentis*. In the light of preliminary enzyme experiments, reported later (Smith, 1960*a*) it seemed unlikely that *S. recurrentis* possesses enzymes of the Entner–Doudoroff pathway (Entner & Doudoroff, 1952) found in some bacteria (Gunsalus, Horecker & Wood, 1955), so that the overall picture provided by these studies is that the spirochaete of relapsing fever possesses a phosphorylative type of metabolism based on anaerobic glycolysis. The final proof of this belief is provided by the enzymic experiments described in the next papers (Smith, 1960*a*, *b*).

SUMMARY

1. The metabolism of *Spirochaeta recurrentis*, the parasite of relapsing fever, has been studied *in vitro* and *in vivo*.

2. Large quantities of lactic acid produced in the blood of the rat host result in a raised output of respiratory carbon dioxide at the peak of infection which is accompanied by hypoglycaemia and depletion of liver glycogen.

3. Experiments *in vitro* have shown that there is no aerobic respiration and that the spirochaetes catabolize glucose by a glycolytic mechanism predominantly to lactic acid, which is accompanied by a small amount of pyruvate. In addition very small amounts of glycerol, *n*-butyric acid, normal and *iso*valeric acids are formed.

4. Inhibition studies of metabolism by fluoride, iodoacetate and azide and results obtained with [U-^{14}C]- and [1-^{14}C]-glucose have confirmed that metabolism of the organisms was of a glycolytic nature.

5. No carbon dioxide attributable to the organisms themselves was given out during catabolism *in vitro* of either of the labelled glucose samples.

6. No incorporation of radioactive label occurs in the isolated organisms after incubation with labelled substrate.

7. The Embden–Meyerhof route of anaerobic glycolysis was the only pathway of glucose catabolism *in vitro* detected.

REFERENCES

Altmann, S. M., Crook, E. M. & Datta, S. P. (1951). *Biochem. J.* **49**, lxiii.

Arnstein, H. R. V. & Grant, P. T. (1957). *Progr. Biophys.* **7**, 167.

Barker, S. (1957). In *Methods in Enzymology*, vol. 3, p. 241. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Barker, S. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.

Barron, E. S. G. (1951). *Advanc. Enzymol.* **11**, 201.

Bernstein, I. A. & Wood, H. A. (1957). In *Methods in Enzymology*, vol. 4, p. 561. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Bloom, B. & Stetten, M. R. (1953). *J. Amer. chem. Soc.* **75**, 5446.

Bloom, B., Stetten, M. R. & Stetten, D. W. (1953). *J. biol. Chem.* **204**, 68.

Brand, T. von (1951). *Exp. Parasit.* **1**, 60.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th ed. London: Baillière, Tindall and Cox.

Bumm, E., Appel, H. & Couciero, P. (1933). *Hoppe-Seyl. Z.* **220**, 186.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*. New York: J. Wiley and Sons Inc.

Cavallini, D. & Frontali, N. (1954). *Biochim. biophys. Acta*, **13**, 439.

Christophers, S. R. & Fulton, J. D. (1938). *Ann. trop. Med. Parasit.* **32**, 43.

Claridge, E. A. & Werkman, C. H. (1953). *Arch. Biochem. Biophys.* **47**, 99.

Coleman, R. M. & Brand, T. von. (1957). *J. Parasit.* **43**, 263.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Coxon, R. V. & Robinson, R. J. (1956). *Proc. Roy. Soc.* B, **145**, 232.

Dawes, E. A. & Holms, W. H. (1958). *Biochim. biophys. Acta*, **29**, 82.

de Duve, C. & Hers, H. G. (1957). *Annu. Rev. Biochem.* **26**, 149.

Dixon, M. (1943). *Manometric Methods*, 2nd ed. Cambridge: Cambridge University Press.

Duncan, R. E. B. & Porteous, J. W. (1953). *Analyst*, **78**, 641.

Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.

Feller, D. D., Strisower, E. H. & Chaikoff, I. L. (1950). *J. biol. Chem.* **187**, 571.

Fenyvessy, B. & Scheff, G. (1930). *Biochem. Z.* **221**, 206.

Fulton, J. D. & Spooner, D. F. (1956). *Exp. Parasit.* **5**, 154.

Geiman, Q. M. (1952). *Annu. Rev. Microbiol.* **6**, 299.

Goodwin, T. W. & Williams, G. R. (1952). *Biochem. J.* **51**, 708.

Grant, P. T. & Fulton, J. D. (1957). *Biochem. J.* **66**, 242.

Gunsalus, I. C., Horecker, B. L. & Wood, W. A. (1955). *Bact. Rev.* **19**, 79.

Hindle, E. (1931). *A System of Bacteriology in Relation to Medicine*, vol. 8. London: H.M.S.O.

Horst, K., Mendel, L. B. & Benedict, F. G. (1934). *J. Nutr.* **7**, 277.

Hullin, R. P. & Noble, R. L. (1953). *Biochem. J.* **55**, 289.

James, A. T. & Martin, A. J. P. (1952*a*). *Biochem. J.* **50**, 679.

James, A. T. & Martin, A. J. P. (1952*b*). *Analyst*, **77**, 915.

Karnovsky, M. L., Foster, J. M., Gidez, L. I., Hagerman, D. D., Robinson, C. V., Solomon, A. K. & Villee, C. A. (1955). *Analyt. Chem.* **27**, 852.

Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.

Lardy, H. A. & Phillips, P. H. (1943*a*). *J. biol. Chem.* **148**, 333.

Lardy, H. A. & Phillips, P. H. (1943*b*). *J. biol. Chem.* **148**, 343.

Long, A. G., Quayle, J. R. & Stedman, R. J. (1951). *J. chem. Soc.* p. 2197.

Long, C. (1944). *Biochem. J.* **38**, 447.

McClintock, R. & Lifson, N. (1957). *J. biol. Chem.* **226**, 153.

McKee, R. W. & Geiman, Q. M. (1950). *Fed. Proc.* **9**, 201.

Mann, T. & Lutwak-Mann, C. (1948). *Biochem. J.* **43**, 266.

Masoro, E. J., Felts, J. M., Panagos, S. S. & Rapport, D. (1957). *Arch. Biochem. Biophys.* **68**, 270.

Mendel, B., Bauch, M. & Strelitz, F. (1931). *Klin. Wschr.* **10**, 118.

Mercado, T. I. (1952). *Amer. J. trop. Med.* **1**, 932.

Mercado, T. I. & Brand, T. von. (1954). *Exp. Parasit.* **3**, 259.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Pesez, M. & Ferrero, J. (1957). *Bull. Soc. Chim. biol., Paris*, **39**, 221.

Rendina, G. & Coon, M. J. (1957). *J. biol. Chem.* **225**, 523.

Santer, M. & Ajl, S. (1955). *J. Bact.* **69**, 298, 713.

Scheff, G. (1935). *Zbl. Bakt.* **134**, 35.

Skipper, H. E., Bryan, C. E., White, L. & Hutchinson, O. S. (1948). *J. biol. Chem.* **173**, 371.

Smith, P. J. C. (1958). *Clin. chim. Acta*, **3**, 586.

Smith, P. J. C. (1960*a*). *Biochem. J.* **76**, 500.

Smith, P. J. C. (1960*b*). *Biochem. J.* **76**, 508.

Steinberg, D. & Udenfriend, S. (1957). In *Methods in Enzymology*, vol. 4, p. 425. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques*. Minneapolis: Burgess Publishing Co.

Wood, H. G. (1955). *Physiol. Rev.* **35**, 841.

Yang, W. C. (1957). *Science*, **125**, 1087.

Biochem. J. (1960) **76**, 500

Carbohydrate Metabolism in *Spirochaeta recurrentis*

2. ENZYMES ASSOCIATED WITH DISINTEGRATED CELLS AND EXTRACTS OF SPIROCHAETES*

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In the preceding paper (Fulton & Smith, 1960) it was shown that the only pathway of glucose catabolism in *Spirochaeta recurrentis* is by glycolysis to lactic acid. In this paper the results of experiments are described which show the presence of the enzymes of the Embden–Meyerhof route in disintegrated spirochaetes and cell-free extracts.

EXPERIMENTAL

The following abbreviations are used in this paper: DPN^+, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+, TPNH, oxidized and reduced triphosphopyridine nucleotide; ATP, ADP, adenosine tri- and di-phosphate; EDTA, sodium ethylenediaminetetraacetate.

Preparation of disintegrated cells. The manner in which the parasites were isolated from rat blood has been described by Fulton & Smith (1960). To disintegrate the organisms, the residue obtained from the final centrifuging in the initial isolation procedure was suspended in ice-cold 0·9% sodium chloride (6–7 ml.) and then passed through a Hughes press (Hughes, 1951; Hugo, 1954) at a temperature of −20°. Removal and microscopical examination of the treated suspension revealed no intact organisms. If not required immediately after crushing the solid-frozen 'homogenate' was kept for a short while at −20°. During experiments it was stored at 0°.

Preparation of cell-free extracts. Particulate matter was removed from the homogenates by centrifuging at 0° for 15 min. at 15 000 *g*. This resulted in a nearly clear, almost colourless extract which was diluted, if necessary, with 0·9% sodium chloride before use. In a few experiments the supernatant resulting from centrifuging the homogenate at 120 000 *g* for 10 min. on a Spinco Universal ultracentrifuge (Beckman Instruments Inc., Calif., U.S.A.) was used.

Chemical determinations. Glucose was determined by Nelson's (1944) method. Pyruvic acid was measured by Kachmar & Boyer's (1953) modification of the Friedemann & Haugen (1943) method. Total nitrogen was assayed by the micro-Kjeldahl technique and protein by the Lowry (1957) modification of the Warburg & Christian (1941) spectrophotometric method.

Optical methods of enzyme and enzymic determinations

Pyridine nucleotide coenzyme-linked dehydrogenases, and those enzymes which could be coupled to dehydrogenases, were assayed by measuring the reduction or oxidation respectively of DPN^+ or DPNH, by the method of Warburg & Christian (1943) as modified by Beisenherz *et al.* (1953).

Reaction mixtures, in quartz cuvettes of 1·0 cm. light path, were usually made up to a total volume of 3 ml. Blanks lacking substrate were measured concurrently with the tests in a spectrophotometer type SP. 500 (Unicam Instruments Ltd., Cambridge, England).

Phosphoglucomutase, hexokinase, phosphoglucoisomerase and phosphofructokinase. These were demonstrated by using the appropriate substrates in the presence of DPN^+ and arsenate, and by following the reduction of the coenzyme when spirochaete cell-free extract was used as a source of enzyme.

α-Glycerophosphate dehydrogenase. This was demonstrated by measuring the decrease in extinction which occurs when DPNH is oxidized by dihydroxyacetone phosphate (Beisenherz *et al.* 1953). An equilibrium mixture of triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in equimolar proportions, was used as the source of substrate and the glyceraldehyde 3-phosphate component removed by using an excess of glyceraldehyde phosphate dehydrogenase and DPN^+ in the presence of arsenate. When the reaction had ceased, the enzyme mixture to be tested was added and the reoxidation of the DPNH followed.

Glyceraldehyde 3-phosphate dehydrogenase. The purified crystalline enzyme and demonstration of activity in extracts was assayed by the Cori, Slein & Cori (1948) method, with triose phosphate equilibrium mixture as substrate in the presence of DPN^+ and arsenate.

3-Phosphoglycerate kinase. The reverse reaction was measured by using 3-phospho-D-glycerate as substrate and by coupling the 1:3-diphospho-D-glycerate formed in the presence of ATP to excess of glyceraldehyde phosphate dehydrogenase (Bücher, 1955). Cysteine was used to trap the glyceraldehyde 3-phosphate produced. The reaction was followed by measuring DPNH oxidation.

Phosphoglyceromutase, enolase and pyruvate kinase. The method was essentially similar to that used by Bücher & Pfleiderer (1955) to assay pyruvate kinase, except that 3-phospho-D-glycerate was used as substrate instead of phosphoenolpyruvate. The pyruvate formed in the reaction was coupled by added lactic dehydrogenase to DPNH and the rate of reaction followed optically.

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Pyruvate kinase. The compound optical assay adapted from Bücher & Pfleiderer (1955), in which phosphoenolpyruvate served as substrate, was used.

Lactic dehydrogenase. Routine assay of the pure preparation, and of the activity of the enzyme in extracts, was performed according to Beisenherz *et al.* (1953), after Kubowitz & Ott (1943). Triethanolamine buffer was used instead of bicarbonate buffer.

Chemical methods of enzyme and enzymic determinations

Aldolase. Activity of this enzyme was measured also by the Bard & Gunsalus (1950) modification of the Sibley & Lehninger (1949) procedure. Colour development was carried out by placing portions (1 ml.) of the deproteinized incubation medium in 10 ml. graduated tubes. 0·75 N-Sodium hydroxide (1 ml.) was added, and the tubes were incubated for 10 min. at 37°, followed by 1 ml. of 0·1% 2:4-dinitrophenylhydrazine and incubation for a further 15 min. Tube contents were then made up to 10 ml. with 0·75 N-sodium hydroxide, shaken and, after allowing to stand for 10 min., their colour was read on a Hilger (Biochem) absorptiometer with an Ilford OG 1 green filter (540 mμ).

Triose phosphate isomerase. The inferred presence of this enzyme in extracts was confirmed by a method based on the addition of hydrazine-trapping agents at the end, instead of at the beginning, of the reaction period in the system used for aldolase determination. Theoretical aspects of the procedure have been discussed by Meyerhof (1951).

Phosphoglyceromutase, enolase and pyruvate kinase. Pyruvic acid, formed as a result of the combined action of the three enzymes when 3-phospho-D-glycerate was used as a substrate, was used as a measure of activity. It was assumed that, in the presence of iodoacetate and absence of added DPNH, depletion of substrate and conversion of pyruvate into lactate were minimal.

Electrode methods of enzyme and enzymic determinations

Hexokinase. Production of 1 equivalent of acid during the initial phosphorylation of glucose at neutral pH was used as the basis of a microtitrimetric constant-pH method for the demonstration of hexokinase activity. The apparatus used was essentially as described by Schwartz & Meyers (1958). Constancy of pH was maintained by addition of standard alkali from a calibrated micrometer syringe (Agla brand, Burroughs Wellcome Ltd., London).

Materials

General reagents. AnalaR-grade chemicals were used, further purification not being attempted unless necessary.

Substrates. All sugars were purchased from British Drug Houses Ltd. (Poole, Dorset, England), as were also D-glucose 6-phosphate (sodium salt), DL-lactic acid and EDTA (sodium salt). D-Fructose 6-phosphate, D-glucose 1-phosphate and 3-phosphoglyceric acid as the barium salts, and the silver–barium salt of phosphoenolpyruvic acid, were purchased from Boehringer und Söhne (Mannheim, West Germany). Sodium pyruvate and triose phosphate equilibrium mixture, consisting of equal quantities of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in solution, were also purchased from the same source. Samples were assayed before use. The barium and calcium salts of D-fructose 1:6-diphosphate were purchased from British Drug Houses Ltd. and L. Light and Co. Ltd. respectively. The barium salt of 6-phosphogluconic acid was prepared by bromine oxidation of D-glucose 6-phosphate (Horecker, 1957).

Buffer solutions. Veronal–acetate buffer was prepared according to Michaelis (1931) and veronal–HCl after Michaelis (1930). Triethanolamine–HCl and bicarbonate–CO_2 buffers were those of Beisenherz *et al.* (1953). Pyrophosphate buffer was made as detailed by Warburg & Christian (1938).

Enzymes. Crystalline aldolase from rabbit muscle was prepared by the Taylor, Green & Cori (1948) procedure. Recrystallization to the stage where no triose phosphate isomerase was present was not attempted. Glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase, both from muscle, were obtained as crystalline suspensions from Boehringer und Söhne. Yeast hexokinase type II (Kunitz & McDonald, 1946) and Zwischenferment, both from Sigma Chemical Co. (St Louis, Mo., U.S.A.) were gifts.

Coenzymes. DPN^+ and DPNH, of the purest quality obtainable, were from Boehringer und Söhne. TPN^+ was a gift. ADP and ATP (sodium salts) were purchased from Sigma Chemical Co. All samples of ATP were assayed with yeast hexokinase before use.

Preparation of solutions. Solutions were prepared by dissolving in ion-exchange-purified water, neutralizing if necessary and making up to the desired volume. Salts containing heavy metals were prepared for use by conversion into their sodium salts with Zeo-Karb 225 cation-exchange resin (The Permutit Co. Ltd., London). With the silver–barium salt of phosphoenolpyruvate (Baer & Fischer, 1949), however, the sodium salt, in solution, was prepared in the manner described by Wold & Ballou (1957).

Substances such as DPN^+ and TPN^+, and like ATP and carbohydrate phosphate esters, which are unstable unless proper precautions are taken (Schlenk, 1951), were stored at −2° in brown-glass bottles over fused calcium chloride and pellets of potassium hydroxide. Solutions were frozen solid and stored for short periods at −10°. Enzyme preparations, stored either as crystalline suspensions in ammonium sulphate or in the form of dry powder, were kept at −2°.

RESULTS

Initial experiments on the reduction of diphosphopyridine nucleotide by spirochaetal preparations. Demonstration of the enzymes aldolase, triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase was based on the composite reactions of the respective enzymes involved when fructose 1:6-diphosphate was used as substrate. Because the reaction was followed by means of the reduction of DPN^+ during the terminal conversion of 3-phospho-D-glyceraldehyde into 1:3-diphospho-D-glycerate this method is called, for convenience of description, the DPN^+-reduction system.

Difficulty was experienced at first in demonstrating activity owing to the presumed presence of heavy-metal ions. This was overcome by the use of

EDTA in the reaction mixture. Typical results obtained with spirochaete extract and fructose 1:6-diphosphate showed that reduction of DPN^+ is nearly linear with respect to time.

The same rate of DPN^+ reduction was obtained under standard conditions with homogenates as with the cell-free extracts, indicating that the enzymes of this system were present in the soluble fraction of the organism. The optimum pH for the DPN^+-reduction system was found to be between pH 8 and 9. The reaction velocity fell off more rapidly on the acid than on the alkaline side of the optimum. EDTA was inhibitory in concentrations above 2 mM. If EDTA at, or just below, this optimum concentration was left in contact with the cell-free extract for a period exceeding a few minutes, and in the absence of substrate, the DPN^+-reduction system was completely inactivated.

The effects of such treatments as change in temperature, storage time and freeze-drying upon homogenates and cell-free extracts were all tried when standard conditions for optical assay had been perfected. Warming of the cuvette contents produced an increase in the initial reaction rate, whereas cooling achieved the reverse effect. Cell-free extracts were normally stored at $-2°$ and retained their activity with respect to the DPN^+-reduction system for at least 5 days. Homogenates, on the other hand, tended to lose some of their enzymic activity in about a day, but if freeze-dried shortly after preparation could be stored in the cold almost indefinitely.

Generally, it was found that with standard preparations of spirochaetal suspension 0·10 ml. of extract contained sufficient enzyme to catalyse the DPN^+-reducing system used in these experiments, the reaction velocity being proportional to the amount of protein up to a concentration of 0·3–0·4 mg./0·1 ml. Under standard-assay conditions the enzymes present were saturated with respect to substrate at a fructose 1:6-diphosphate concentration greater than about 3·3 mM.

Effect of cysteine and glutathione. On the basis of their power to catalyse the DPN^+-reduction system it was thought highly probable that cell-free extracts of *S. recurrentis* contained glyceraldehyde phosphate dehydrogenase. As this enzyme and others like it are protected and their activities are enhanced by contact with sulphydryl-group-containing compounds (Cori *et al.* 1948; Taylor *et al.* 1948), the effects of both cysteine and glutathione on the spirochaetal extracts were tried. Cysteine (see Table 1) was able to replace EDTA entirely and strongly enhanced the activity when the extract was preincubated in its presence. Glutathione was able to do likewise, but to a less extent. It still activated the extract when added

Table 1. *Effect of cysteine and glutathione on reduction of diphosphopyridine nucleotide in the presence of fructose* 1:6-*diphosphate by cell-free extracts of* Spirochaeta recurrentis

Reaction mixture contained: 1·5 ml. of acetate–veronal buffer, 0·14 M, pH 8·0; 0·1 ml. of DPN^+, 4 mg./ml.; 0·5 ml. of arsenate, 50 μmoles; 0·1 ml. of enzyme preparation; 0·1 ml. of fructose diphosphate, 12 μmoles; 0·1 ml. additions, 8 μmoles; water to 3 ml. Pre-incubation was performed at room temperature for 20 min. before addition of substrate. Substrate was added at zero time. Blanks contained water to replace substrate.

Additions	Activity (ΔE min.$^{-1}$. mg. of protein^{-1}, 340 mμ)	
	Not pre-incubated	Pre-incubated
EDTA	0·039	0·001
Glutathione	0·297	0·278
Cysteine	0·394	0·561
EDTA + glutathione	0·139	0·054
EDTA + cysteine	0·002	0·006

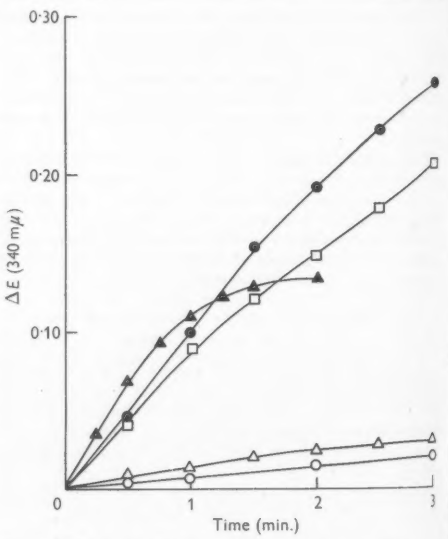

Fig. 1. Effect of different glycolytic intermediates on reduction of DPN^+. Cuvettes contained: 1·5 ml. of veronal–acetate buffer, 0·14 M, pH 8·0; 0·1 ml. of DPN^+, 4 mg./ml.; 0·5 ml. of sodium arsenate, 50 μmoles; 0·1 ml. of EDTA, 8 μmoles. The following substrates, 10 μmoles each, were used: ○, D-glucose 1-phosphate; △, D-glucose 6-phosphate; □, D-fructose 6-phosphate; ●, D-fructose 1:6-diphosphate; ▲, triose phosphate equilibrium mixture containing 3-phospho-D-glyceraldehyde and dihydroxyacetone phosphate in equimolar amounts. Reaction was started by adding cell-free extract, containing 220 μg. of protein/0·1 ml. added. Temp. 20°. Blanks contained water instead of substrate.

with EDTA, whereas cysteine plus the chelating agent was almost totally inhibitory.

Effect of glycolytic intermediates. When the phosphorylated sugars D-glucose 1-phosphate, D-glucose 6-phosphate and D-fructose 6-phosphate were used as substrates in equimolar amounts their effectiveness was related to the number of chemical transformations they had to undergo before reaching the ultimate dehydrogenating step, at the level of 3-phospho-D-glyceraldehyde. A substrate solution consisting of an equilibrium mixture of the two triose phosphate esters, 3-phospho-D-glyceraldehyde and dihydroxyacetone phosphate, caused the most rapid reduction of DPN^+, but its initial effectiveness was short-lived. These results are shown in Fig. 1.

Glucose by itself was not utilized when incorporated into the test system, but when it was added in the presence of ATP and Mg^{2+} ions a slow but sustained reduction of DPN^+ was brought about. Gluconic acid 6-phosphate was completely without effect on the system when used as substrate in the presence of either DPN^+ or TPN^+. D-Glucose 6-phosphate was inactive in the presence of TPN^+, but not, as shown earlier, with DPN^+. These results were indicative of the presence of hexokinase, phosphoglucoisomerase, phosphofructokinase, aldolase and phosphoglyceraldehyde dehydrogenase.

Demonstration of individual glycolytic enzymes in spirochaetal preparations

Hexokinase. The ability of whole spirochaetes to metabolize glucose readily and the catalysis of the reduction of DPN^+ by cell-free extracts in the presence of ATP and Mg^{2+} ions provided evidence for the existence of an active hexokinase. However, demonstration of this enzyme proved to be difficult until microtitrimetric methods were tried, in particular one described by Schwartz & Meyers (1958). Fig. 2 shows *S. recurrentis* hexokinase activity in homogenate and cell-free extract. As can be seen, most of the activity appears to reside in the particulate portion of the organisms.

The reaction mixture required EDTA and though cysteine could be substituted for it there was no activation in its presence. Despite the use of comparatively large amounts of homogenate the reaction proceeded slowly by comparison with the DPN^+-reduction system. Once thawed, the preparation retained its activity only for a few hours.

Aldolase. As shown in Fig. 3, the splitting of fructose 1:6-diphosphate catalysed by the

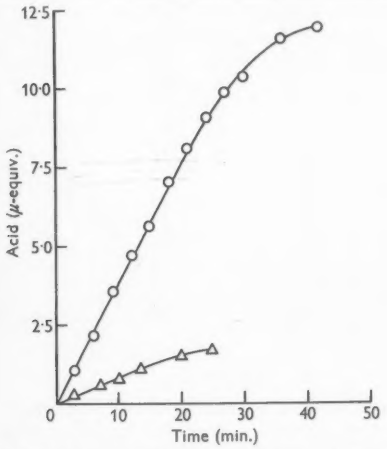

Fig. 2. Hexokinase activity in *S. recurrentis*. Reaction mixture contained: 0·1 ml. of KH_2PO_4, 10 μmoles; 0·1 ml. of EDTA, 8 μmoles; 0·5 ml. of ATP, 20 μmoles; 0·3 ml. of $MgSO_4,7H_2O$, 45 μmoles; 0·2 ml. of glucose, 160 μmoles; 1–2 ml. of homogenate, approx. 9 mg. dry wt./ml. Water to 4 ml. Cell-free extract was prepared by centrifuging a portion of homogenate at 120 000 *g* for 10 min. Reaction was started by adding substrate. Temp. 35°. pH was maintained at 7·50 by titration with standard alkali (0·1 N-NaOH). Blank runs were performed in the absence of substrate. ○, With complete homogenate; △, with cell-free extract.

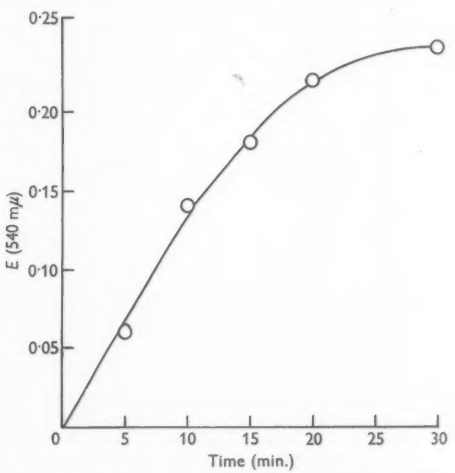

Fig. 3. Time course of aldolase activity. Assay tubes contained: 1·5 ml. of veronal–acetate buffer, 0·14 M, pH 8·5; 0·25 ml. of 0·56 M-hydrazine sulphate, pH 7·6; 0·20 ml. of cell-free extract, diluted with 0·9% NaCl to contain 25 μg. of protein; 0·20 ml. of fructose 1:6-diphosphate, 8 μmoles; water to 2·5 ml. Temp. 37°. Substrate was added at zero time. Blank contents were as given above but substrate was added after addition of 2 ml. of 10% (w/v) trichloroacetic acid to each tube to stop the reaction. Tubes were cooled in ice.

spirochaete extract, plotted as extinction of the chromogens produced from the triose phosphates versus time, followed a nearly linear course for the first 10–15 min. at pH 8·5. Cell-free extracts prepared in a standard manner (see Experimental section) were found to be so active that it was necessary to dilute them 10–20 times with ice-cold 0·9% sodium chloride before use.

Freeze-dried homogenates of *S. recurrentis*, stored at 0°, retained their aldolase activity for at least 2 weeks and when extracted with ice-cold 0·9 % sodium chloride furnished a supply of experimental material for use when fresh organisms were unavailable, but the activity of extracts fell with storage.

Triose phosphate isomerase. The inferred presence of this enzyme was derived from the modified aldolase-assay system. Table 2 gives the results of a typical experiment, in which, from the figures quoted, the colour intensity of one set of tubes, to which hydrazine had been added last, was twice that of another which contained the trapping agent initially. This effect is ascribed to the action of triose phosphate isomerase and it was concluded therefore that the enzyme is present in cell-free extracts of *S. recurrentis*.

D-*Glyceraldehyde* 3-*phosphate dehydrogenase.* Preliminary experiments on the DPN^+-reducing

Table 2. *Triose phosphate isomerase in* Spirochaeta recurrentis

Tube contents and colour development were as described in the Experimental section, except that hydrazine was added before or after trichloroacetic acid, as indicated in the Table. Both sets of tubes contained 50 μg. of protein each and 15 μmoles of fructose diphosphate; pH 8·5. Temp. 37°.

	E (540 mμ)	
Time (min.)	Hydrazine added before acid	Hydrazine added after acid
5	0·075	0·14
10	0·145	0·28
15	0·170	0·34

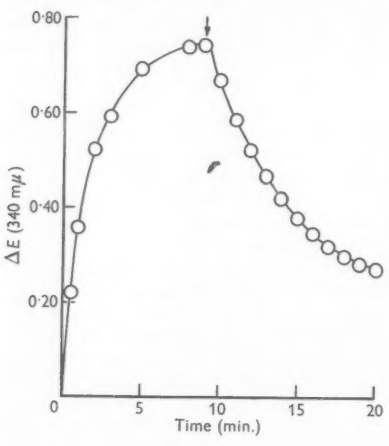

Fig. 4

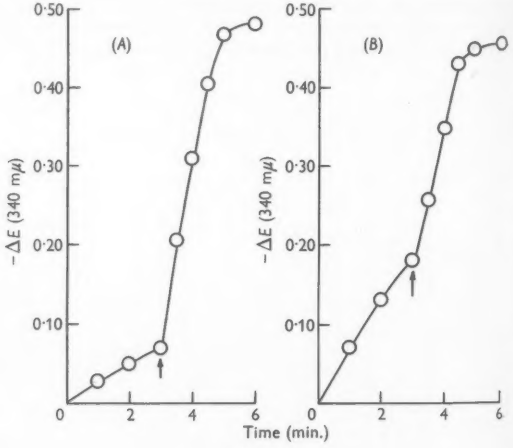

Fig. 5

Fig. 4. α-Glycerophosphate-dehydrogenase activity in *S. recurrentis*. Cuvette contained: 1·8 ml. of triethanolamine buffer, 0·05 M, pH 7·5; 0·1 ml. of DPN, 4 mg./ml.; 0·5 ml. of arsenate, 50 μmoles; 0·05 ml. of triose phosphate equilibrium mixture, containing 5 μmoles each of 3-phospho-D-glyceraldehyde and dihydroxyacetone phosphate; 0·1 ml. of cysteine, 8 μmoles; 20 μg. of pure muscle glyceraldehyde phosphate dehydrogenase; water to 2·8 ml. Blank contained all except substrate. Reaction was started at zero time by addition of glyceraldehyde phosphate dehydrogenase. When the reaction had stopped, 0·2 ml. of cell-free extract, containing 400 μg. of protein, was added at the time marked by the arrow to test and blank cuvettes. Temp. 20°.

Fig. 5. Effect of added ATP and Mg^{2+} ions on phosphoglycerate kinase of *S. recurrentis*. Cuvettes contained: 2·0 ml. of sodium bicarbonate, 50 μmoles; 0·05 ml. of ATP, 5 μmoles; 0·25 ml. of cysteine, 60 μmoles; 0·25 ml. of glycine, 8 mg./ml.; 0·05 ml. of DPNH, 10 mg./ml.; 0·10 ml. of $MgSO_4,7H_2O$, 15 μmoles; 0·10 ml. of 3-phospho-D-glycerate, 15 μmoles; 0·02 ml. of pure muscle glyceraldehyde phosphate dehydrogenase, 10 mg./ml.; water to 2·8 ml. Mixture was saturated with 10 % CO_2 and reaction started by adding 0·2 ml. of cell-free extract, 50 μg. of protein. (*A*) ATP omitted, then added at the time marked with an arrow; (*B*) Mg^{2+} ions omitted, then added at the time marked with an arrow.

system (see above) showed the rate of reduction of coenzyme to be greatest with triose phosphate as substrate (Fig. 2), but that the reaction rapidly came to a standstill even when addition of pure muscle glyceraldehyde phosphate dehydrogenase to the reaction mixture showed the system to be working perfectly. Repeated experiments confirmed the initial findings and showed that inclusion of cysteine in the reaction mixture caused a slow reoxidation of the DPNH formed. Control tests illustrated that the effect was truly a property of the cell-free extract. The presence of a α-glycerophosphate dehydrogenase in the extracts could explain this phenomenon and tests for its presence were therefore undertaken.

α-Glycerophosphate dehydrogenase. As it appears that the particulate type of α-glycerophosphate dehydrogenase is localized within cell mitochondria and associated with the cytochrome system (Green, 1936; Dewan & Green, 1937) it was presumed that the spirochaetal enzyme would be present in the soluble extracts of the organism. The 3-phospho-D-glyceraldehyde component of the triose phosphate equilibrium mixture used as a source of substrate was selectively removed by using excess of pure glyceraldehyde phosphate dehydrogenase in the reaction mixture, leaving, predominantly, dihydroxyacetone phosphate. On addition of a comparatively small amount of cell-free extract a rapid reoxidation of the DPNH, formed initially, occurred (Fig. 4). The velocity of reaction was proportional to the amount of extract added.

Phosphoglycerate kinase. Cell-free extracts of *S. recurrentis* contained phosphoglycerate kinase, the activity of which was dependent upon the presence of ATP and Mg^{2+} ions. As may be seen in Fig. 5, absence of added ATP had a more marked effect upon activity than omission of Mg^{2+} ions.

Phosphoglycerate mutase, enolase and pyruvate kinase. Two methods were used for the demonstration

Table 3. *Inhibition of pyruvate production from 3-phospho-D-glycerate by fluoride*

Tubes contained: 2 ml. of veronal–acetate buffer, 0·14 M, pH 8·0; 0·1 ml. of sodium iodoacetate, 10 μmoles; 0·05 ml. of EDTA, 4 μmoles; 0·1 ml. of $MgCl_2$, 10 μmoles; 0·1 ml. of ADP, 5 μmoles; 0·2 ml. of cell-free extract, containing 0·4 mg. of protein; 0·1 ml. of 3-phospho-D-glycerate, 10 μmoles. One set of tubes contained 0·1 ml. of potassium fluoride, 50 μmoles. After pre-incubation for 10 min., substrate was added to start reaction. Blanks contained no substrate. Temp. 37°.

Time (min.)	Pyruvate (μmoles)	
	Fluoride absent	Fluoride present
10	0·35	0·02
20	1·00	0·04
30	2·00	0·08
40	2·46	0·08
50	2·96	0·08

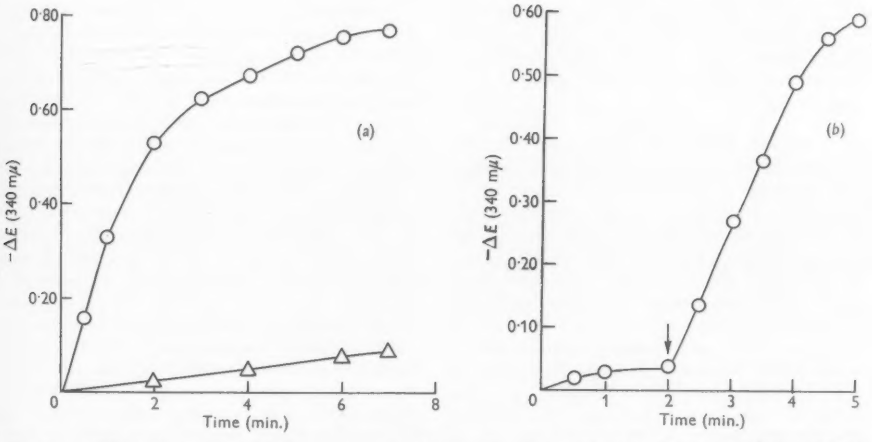

Fig. 6. (*a*) Effect of absence of ADP on pyruvate kinase. Cuvettes contained: 2·0 ml. of triethanolamine buffer, 0·05 M, pH 7·5; 0·15 ml. of 0·5 M-$MgSO_4,7H_2O$ in 2 M-KCl; 0·10 ml. of ADP, 5 μmoles; 0·05 ml. of DPNH, 10 mg./ml.; 0·02 ml. of pure muscle lactic dehydrogenase, 5 mg./ml.; 0·2 ml. of cell-free extract, 0·7 mg. of protein; 0·5 ml. of phosphoenolpyruvate, 6 μmoles. Reaction was started by adding substrate at zero time. Blank contained water. Temp. 20°. ○, Complete reaction mixture; △, no added ADP. (*b*) Effect of Mg^{2+} and K^+ ions on pyruvate kinase of *S. recurrentis*. Cuvette contents were as given in Fig. 6 (*a*). Mg^{2+} (75 μM) and K^+ (0·3 mM) ions were added at the time indicated by the arrow, instead of initially.

of this chain of enzymes, both based on measurement of pyruvate produced when 3-phospho-D-glycerate was used as substrate. The systems contained buffer, EDTA, Mg^{2+} ions, ADP and extract, glyceraldehyde phosphate dehydrogenase activity being inhibited by including iodoacetate. Preliminary experiments having shown the presence of a DPN^+-linked lactic dehydrogenase in the extracts, it was felt that in the absence of added DPNH, transformation of the pyruvate formed into lactate would be prevented. Table 3 shows the time course of pyruvic acid production catalysed by the *S. recurrentis* extract and its inhibition by fluoride. Values for the uninhibited system plotted graphically reveal a time lag, before the attainment of maximal velocity, which was largely abolished by pre-incubation of the reaction mixture in both optical and chemical assays, before addition of substrate. When the pyruvate produced in the reaction was measured by following DPNH oxidation, coupled to added pure lactic dehydrogenase, essentially similar results were obtained. Incubation of the extract with ADP and Mg^{2+} and K^+ ions was found to be necessary for maximal activity.

Pyruvate-kinase activity was demonstrated also in cell-free extracts and was dependent upon the presence of ADP and metal ions. The results are summarized in Fig. 6 (*a* and *b*).

Lactic dehydrogenase. Cell-free extracts of *S. recurrentis* possessed lactic-dehydrogenase activity, and this enzyme forms the subject of a later paper (Smith, 1960*b*). Activity was not destroyed by freeze-drying homogenates of the organism, and closely paralleled that of fresh material when reconstituted with 0·9 % sodium chloride.

DISCUSSION

The presence of enzymes of the glycolytic pathway of glucose metabolism in extracts of *S. recurrentis* has been shown in this series of experiments. Except for hexokinase, these enzymes are located exclusively in the non-sedimentable portion of the organism. Rigid exclusion of formed blood elements, which are known to contain adenosine triphosphatase (Caffrey, Tremblay, Gabrio & Huennekens, 1956) and hexose monophosphate oxidative-cycle enzymes (Bruns, Noltmann & Vahlhaus, 1958), has shown further that the enzymes in spirochaetal homogenates are predominantly glycolytic. Indeed, the suitability of phosphorylated sugars as substrates in the DPN^+-reduction system indicates the ability of these spirochaetes to participate in conventional reactions of glycolysis. Absence of reaction with D-glucose 6-phosphate and 6-phosphogluconate, coupled to TPN^+, is to be expected in an organism without either the hexose monophosphate pathway or the Entner–Doudoroff cleavage (Entner & Doudoroff, 1952).

The 'key' nature of the aldolase reaction in micro-organisms (Gunsalus, Horecker & Wood, 1955) and the ready utilization here of fructose 1:6-diphosphate as a substrate in the DPN^+-reduction system suggested that this enzyme plays a significant part in the carbohydrate metabolism of *S. recurrentis*. This was borne out by the fact that the organisms are rich in aldolase activity.

Enzymes present in cell-free extracts of *S. recurrentis* are susceptible to inhibition by heavy metals, as judged by their behaviour towards EDTA. The inhibition produced by large amounts of EDTA suggests activation by metal ions. Enhancement of enzymic activity in the presence of sulphydryl compounds, over and above that attributable to removal of heavy metals, indicates that some of the enzymes present may rely on the presence of intact sulphydryl groups for maximal activity, as has been shown to be the case with muscle glyceraldehyde phosphate dehydrogenase (Cori *et al.* 1948; Taylor *et al.* 1948). The completely inhibitory effect of cysteine and EDTA combined may possibly be caused by the high stabilities of their metal chelates (Albert, 1952; Martell & Calvin, 1952) and consequent inactivation of the enzymes by withdrawal of all metal ions from the system. However, as these were crude extracts, the conclusions from such experiments must be necessarily tentative.

In addition to an active aldolase, cell-free extracts also contain triose phosphate isomerase. This is hardly surprising, since isomerase accompanies aldolase in all tissues and is present in much higher concentration (Meyerhof & Beck, 1944). Baernstein & Rees (1952) also showed the presence of the enzyme in trypanosome extracts by methods similar to those used in these experiments.

Detection of glycerol in traces during spirochaete metabolism is explained by the presence in them of a DPN^+-dependent α-glycerophosphate dehydrogenase. There seems no doubt also that glyceraldehyde phosphate dehydrogenase is present, but with the substrate available the reaction equilibrium was unfavourable for its demonstration.

The remaining enzymes of the glycolytic sequence which were demonstrated appear to resemble those from animal or plant sources. Phosphoglycerate kinase, like the enzyme from yeast, requires adenine nucleotide coenzyme and Mg^{2+} ion for maximal activity. The time lag before development of maximal activity in the composite reaction catalysed by the enzymes phosphoglycerate mutase, enolase and pyruvate kinase might reflect a requirement for 2:3-diphospho-D-glycerate in the initial phosphate-transfer reaction. As fluoride is

an inhibitor of enolase (Warburg & Christian, 1941), it is very probable that the inhibition observed in these experiments is upon this enzyme. Lohmann & Meyerhof (1934) found that the activity of pyruvate kinase from muscle is linked to the presence of bivalent ions such as magnesium, and later (Boyer, Lardy & Phillips, 1942) the requirement for univalent ions such as K^+ was demonstrated. The enzyme from *S. recurrentis* also is inactive without these two ions.

The almost universal occurrence of lactate and pyruvate as metabolic products indicates a nearly ubiquitous distribution of lactic dehydrogenase (Schlenk, 1951). *S. recurrentis* possesses this enzyme and doubtless it plays an important part in the metabolic economy of the organism. The effectiveness of DPNH in the reaction catalysed by spirochaetal extracts leads to the conclusion that this is the natural coenzyme, as in muscle and heart, in contrast with the cytochrome b_2-linked lactic dehydrogenase found in yeast (Bach, Dixon & Zerfas, 1946).

Therefore from the results of this and preceding studies, the carbohydrate metabolism of *S. recurrentis* can be said to be solely glycolytic. The complete scheme of carbohydrate metabolism in microorganisms, even for known pathways, is often not documented for a single species or even for a single genus, this applying particularly to the protozoa and the spirochaetes, and tacit assumption by past workers of the existence of a particular pathway has often been based on fermentations studied only at substrate to product level. Consequently it is believed that the three main lines of inquiry, represented by the sections of this work done *in vivo* and *in vitro* (Fulton & Smith, 1960) and by the enzymic experiments reported here, constitute the first elucidation of a conventional pathway of intermediary metabolism in any member of the order Spirochaetales. The further properties of some of the more important enzymes concerned in the carbohydrate metabolism of *S. recurrentis* are described in the following papers (Smith, 1960*a*, *b*).

SUMMARY

1. Homogenates and cell-free extracts of *Spirochaeta recurrentis* have been shown to contain enzymes of the Embden–Meyerhof pathway of glycolysis.

2. The following enzymes have been demonstrated both in combination and independently. Hexokinase, phosphoglucoisomerase, phosphofructokinase, aldolase, phosphoglyceraldehyde dehydrogenase, triose phosphate isomerase, oxidized diphosphopyridine nucleotide dependent α-glycerophosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, pyruvate kinase and oxidized diphosphopyridine nucleotide-dependent lactic dehydrogenase.

3. The general properties of these enzymes appear to resemble those from other sources. Hexokinase activity resides in the particulate portion of the organisms, whereas the other enzymes are soluble. At least some of them require metal ions and intact sulphydryl groups for full activity.

The author is gratefully indebted to Dr J. D. Fulton, in whose Department this work was performed, and to Dr P. Greengard for gifts of enzymes. Thanks are also due to Mr L. Hills for maintaining the organism.

REFERENCES

Albert, A. (1952). *Biochem. J.* **50**, 690.
Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.
Baer, E. & Fischer, H. O. L. (1949). *J. biol. Chem.* **180**, 145.
Baernstein, H. D. & Rees, C. W. (1952). *Exp. Parasit.* **1**, 215.
Bard, R. C. & Gunsalus, I. C. (1950). *J. Bact.* **59**, 387.
Beinsenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8***b*, 555.
Boyer, P. D., Lardy, H. A. & Phillips, P. H. (1942). *J. biol. Chem.* **146**, 673.
Bruns, F. H., Noltmann, E. & Vahlhaus, E. (1958). *Biochem. Z.* **330**, 483.
Bücher, Th. (1955). In *Methods in Enzymology*, vol. 1, p. 415. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Bücher, Th. & Pfleiderer, G. (1955). In *Methods in Enzymology*, vol. 1, p. 435. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Caffrey, R. W., Tremblay, R., Gabrio, B. W. & Huennekens, F. M. (1956). *J. biol. Chem.* **223**, 1.
Cori, G. T., Slein, M. W. & Cori, C. F. (1948). *J. biol. Chem.* **173**, 605.
Dewan, J. G. & Green, D. E. (1937). *Biochem. J.* **31**, 1074.
Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Fulton, J. D. & Smith, P. J. C. (1960). *Biochem. J.* **76**, 491.
Green, D. E. (1936). *Biochem. J.* **30**, 629.
Gunsalus, I. C., Horecker, B. L. & Wood, W. A. (1955). *Bact. Rev.* **19**, 79.
Horecker, B. L. (1957). In *Methods in Enzymology*, vol. 3, p. 172. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Hugo, W. B. (1954). *Bact. Rev.* **18**, 87.
Kachmar, J. E. & Boyer, P. D. (1953). *J. biol. Chem.* **200**, 669.
Kubowitz, F. & Ott, P. (1943). *Biochem. Z.* **314**, 94.
Kunitz, M. & McDonald, K. (1946). *J. gen. Physiol.* **29**, 393.
Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.
Lowry, O. H. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall Inc.
Meyerhof, O. (1951). In *The Enzymes*, vol. 2, part 1, p. 162. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Meyerhof, O. & Beck, L. V. (1944). *J. biol. Chem.* **156**, 109.
Michaelis, L. (1930). *J. biol. Chem.* **87**, 33.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Schlenk, F. (1951). In *The Enzymes*, vol. 2, part 1, p. 250. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Schwartz, M. & Meyers, T. C. (1958). *Analyt. Chem.* **30**, 1150.
Sibley, J. A. & Lehninger, A. L. (1949). *J. biol. Chem.* **177**, 859.
Smith, P. J. C. (1960*a*). *Biochem. J.* **76**, 508.
Smith, P. J. C. (1960*b*). *Biochem. J.* **76**, 514.
Taylor, J. F., Green, A. A. & Cori, G. T. (1948). *J. biol. Chem.* **173**, 591.
Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
Warburg, O. & Christian, W. (1943). *Biochem. Z.* **314**, 94.
Wold, F. & Ballou, C. E. (1957). *J. biol. Chem.* **227**, 302.

Biochem. J. (1960) **76**, 508

Carbohydrate Metabolism in *Spirochaeta recurrentis*

3. PROPERTIES OF ALDOLASE IN SPIROCHAETES*

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The enzyme aldolase, which catalyses the reversible splitting of fructose diphosphate, is of universal distribution amongst those living things which catabolize glucose by the Embden–Meyerhof route (Stumpf, 1954). In fact, since aldolase constitutes a 'key' enzyme in the reactions of anaerobic glycolysis its existence has been used as an indication of the presence of this pathway in various micro-organisms (Gunsalus, Horecker & Wood, 1955).

Recently, interest has been focused upon properties of the enzyme illustrated by the work of Rose & Rieder (1958) and the studies of Krishna Murti (1958), which have been concerned respectively with the mechanism of aldolase action and with its metal-ion activation in muscle, yeast and bacteria. Aldolase activity has been demonstrated in *Spirochaeta recurrentis* (Smith, 1960) and, as nothing is known about this enzyme in spirochaetes, a general study was made of its properties. On the results of work reported here, evidence is presented for the existence of a metallo-aldolase in this micro-organism.

EXPERIMENTAL

Methods

Isolation of spirochaetes from the blood of infected rats and preparation of cell-free extracts have been described by Smith (1960), as have the methods for determination of extinction, nitrogen and protein.

Abbreviations. The following abbreviations have been used in this paper: FDP, D-fructose 1:6-diphosphate; EDTA, ethylenediaminetetra-acetate (sodium salt); PCMB, *p*-chloromercuribenzoate.

Aldolase assay. The purified enzyme prepared from rabbit-skeletal muscle, which was used in FDP determinations, was assayed by the Warburg & Christian (1943) procedure as modified by Taylor (1955).

With crude extracts optical assay cannot be relied upon to provide accurate information, and therefore aldolase activity of spirochaetal extracts was measured by the Bard & Gunsalus (1950) modification of the Sibley & Lehninger (1949) technique as described by Smith (1960). The activity of the extracts was expressed in μg. of alkali-labile phosphorus produced/hr./mg. of protein by using the factor 10·8, after the manner of Bard & Gunsalus (1950) for an exactly similar assay system calibrated with purified FDP.

pH *measurements.* Determination of optimum pH of the spirochaete aldolase was carried out in veronal–acetate buffer (Michaelis, 1931), solutions being checked before use with a meter (Muirhead Type D-417, Muirhead and Co. Ltd., Beckenham, Kent, England) and glass electrode.

Inhibition and activation experiments. Tube contents were pre-incubated in the presence of various inhibitors, and for this purpose EDTA, 8-hydroxyquinoline, $\alpha\alpha'$-dipyridyl and PCMB were added to the aldolase-assay tubes 10 min. before the substrate. Bard & Gunsalus (1950) recommended that tests with dipyridyl be carried out at pH 6·0 because of the greater activity of the reagent as a Fe^{2+}-ion binder at acid pH, but owing to the finding that a fairly sharp alkaline pH optimum existed for *S. recurrentis* aldolase this was not possible. When using cysteine, glutathione or metal ions, alone or in combination, precautions were taken to allow for the complex-forming tendencies which

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metals exhibit with amino acids or peptides (Gilbert, Otey & Price, 1951; Rabin, 1955). In addition, tubes in which metal ions and cysteine were present were gassed with hydrogen, as used by Jagannathan, Singh & Damodaran (1956), to avoid the atmospheric oxidation of Fe^{2+} and Co^{2+} ions.

Dialysis. Dialysis of diluted cell-free extracts was carried out in cellophan tubing (Visking–Nojax, U.S.A.), previously cleaned by boiling with water and soaking according to the method described by Klotz & Hughes (1956). To ensure thorough mixing of the contents glass beads were enclosed in the dialysis sacs.

Materials

General reagents were of AnalaR grade wherever possible. FDP was prepared by yeast fermentation, after the method of Neuberg & Lustig (1942), and purified as the magnesium salt (Neuberg & Sabatay, 1925). This salt was converted into the sodium salt as previously described (Smith, 1960). FDP samples were assayed optically with rabbit-muscle aldolase and glyceraldehyde 3-phosphate dehydrogenase (Cori, Slein & Cori, 1948). Reduced glutathione, dipyridyl, 8-hydroxyquinoline and EDTA (disodium) were commercial preparations. Cysteine hydrochloride and PCMB were gifts.

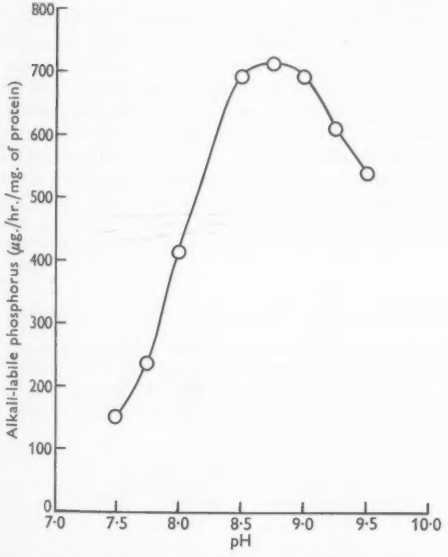

Fig. 1. Effect of pH on aldolase activity. Tubes contained: 1·5 ml. of veronal–acetate buffer, 0·14 M, of pH shown; 0·25 ml. of 0·56 M-hydrazine sulphate; 0·20 ml. of cell-free extract, diluted with 0·9 % NaCl to contain approx. 25 μg. of protein; 0·20 ml. of FDP, 15 μmoles; water to 2·5 ml. Temp. 37°. Substrate was added at zero time. Incubated for 15 min. Blanks had the same contents as listed above, but substrate was added after 2 ml. of 10 % (w/v) trichloroacetic acid had been used to stop the reaction.

RESULTS

Previous experiments (Smith, 1960) have shown that extracts of *S. recurrentis* contain an active diphosphofructoaldolase, and that enzymic activity is located within the soluble portion of the organism. These findings were confirmed and experiments extended to examine the following properties.

Enzyme concentration and activity. The rate of the aldolase-catalysed splitting of FDP was found to be linear over the range 10–60 μg. of protein. For this reason tests were arranged to contain from 10 to 60 μg. of protein/2·5 ml. of reaction mixture.

pH *optimum.* Experiments over the range pH 7·5–9·5 show that the optimum for *S. recurrentis* aldolase lies at or about pH 8·75 (Fig. 1). The activity rises sharply before the optimum and falls off less rapidly thereafter.

Effect of substrate concentration. Examination of the curve showing reaction velocity–FDP concentration (Fig. 2), reveals that under these experimental conditions reaction velocity was maximal at a substrate concentration of about 3 mM. An approximate value for the Michaelis constant derived from the experimental curve (Michaelis & Menten, 1913) is 1·4 mM. It must be emphasized that this value is an approximation, owing to the

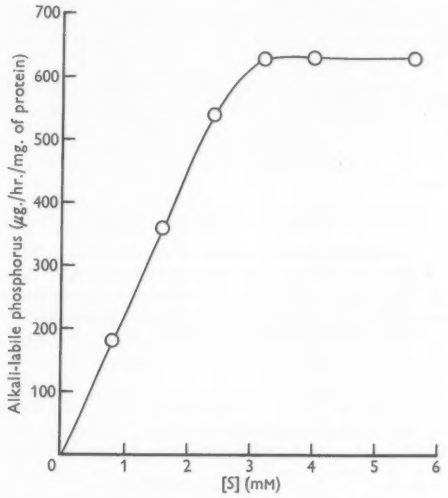

Fig. 2. Effect of substrate concentration on aldolase activity. Tube contents were as described in Fig. 1, except that FDP concentration varied from 2 to 14 μmoles per tube. Velocity of the reaction was measured over 20 min. at pH 8·5 and at 37°. Blanks contained corresponding amounts of FDP, added after the reaction had been stopped with trichloroacetic acid.

unpurified nature of the extracts used. For the same reason demonstration of activity of the spirochaetal enzyme towards other substrates such as fructose 1-phosphate, as has been shown for liver aldolase (Leuthardt & Wolf, 1954), was not attempted.

Effect of metal-binders and activators on aldolase. The requirement of *S. recurrentis* aldolase for metal ions and the presence of intact sulphydryl groups for full activity was investigated by means of metal-complexing agents, sulphydryl reagents and a sulphydryl-group inactivator. Experiments were set up in which the enzyme was exposed to EDTA, 8-hydroxyquinoline, dipyridyl, or to cysteine, glutathione or PCMB (Hellerman, Chinard & Deitz, 1943) as detailed below. The bivalent metal ions Fe^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} also were used.

(*a*) *Effect of metal-binders and sulphydryl reagents.* Table 1 shows the effect on aldolase activity of the metal-ion chelators mentioned above. Included in this table is the effect of pre-incubation of the cell-free extract in the presence of PCMB, and of equivalent amounts of cysteine and glutathione. It can readily be seen that, of the chelating agents, EDTA, an efficient bivalent-cation-binder, inhibited most effectively, whereas 8-hydroxyquinoline and dipyridyl did so to a less extent. PCMB completely inhibited enzyme activity at both the concentrations used, and cysteine and reduced glutathione enhanced the activity considerably; this has been shown to be characteristic of sulphydryl-group-requiring enzymes (Madsen & Cori, 1956). From these results it was concluded that *S. recurrentis* aldolase is probably a sulphydryl enzyme requiring metal ions for full activity.

(*b*) *Effect of bivalent-metal ions.* The ions Fe^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were investigated. These metals were chosen because of the role they frequently play in biological systems (Williams, 1953).

Solutions of the hydrated sulphates of the metals were prepared to give final concentrations of 0·4 mM; earlier tests revealed that double or treble this concentration was too great for the enzyme dilution used and made comparison difficult. Pre-incubation of the enzyme with the metal was a prerequisite for full effect. Table 2 (*a*) shows the results of a typical series of experiments in which the use of metal ions alone demonstrated that Co^{2+} and, to a lesser extent, Zn^{2+} ions activated the enzyme, whereas Fe^{2+} and Mn^{2+} ions were inhibitory. Mg^{2+} ions appeared neither to activate nor to inhibit.

Next (Table 2 *b*) a small amount of EDTA was used to chelate extraneous ions first, then a slight excess of each metal ion was added and the cell-free extract incubated in its presence before the addition of FDP. There was a well-defined activation by Co^{2+} and Zn^{2+} ions, with cobalt again exerting the greater effect. All other metals inhibited in this experiment.

(*c*) Effect of metal ions plus cysteine. The combined effect of metal ions and cysteine upon the system was tried. The order in which cell-free extract, metal ion and cysteine were added, and the quantities, were found to be of the greatest importance in obtaining reproducible results. In the system finally adopted the metal ion was added immediately after the extract and about 10 min. at 37° was allowed for activation to take place, the assumption being that the metal combines first with the protein, as reported by Malmström (1954)

Table 1. *Effect of metal-complexing agents and sulphydryl reagents on* Spirochaeta recurrentis *aldolase*

Tubes contained: 1·5 ml. of veronal–acetate buffer (0·14 M), pH 8·5; 0·25 ml. of 0·56 M-hydrazine sulphate, pH 7·6; 0·20 ml. of cell-free extract, diluted to contain 25 μg. of protein; 0·15 ml. addition, 0·2 ml. of FDP, 15 μmoles; water to 2·5 ml. Tubes were pre-incubated at 37° for 10 min. after making additions. Reaction was started by adding FDP and allowed to proceed for 15 min. Appropriate blanks contained additions but no FDP. Colour development was by the usual method.

Additions	Concn. (mM)	Activity (μg. of alkali-labile phosphorus/ hr./mg. of protein)	Percentage of original activity
None	—	605	100
EDTA	1·6	43	7
EDTA	0·8	46	8
8-Hydroxyquinoline	1·6	173	29
8-Hydroxyquinoline	0·8	432	71
αα′-Dipyridyl	1·6	259	43
αα′-Dipyridyl	0·8	562	93
PCMB	1·6	0	0
PCMB	0·8	0	0
Cysteine	1·6	1510	249
Cysteine	0·8	993	164
Glutathione	1·6	1340	222
Glutathione	0·8	820	135

Table 2. *Effect of metal ions on* Spirochaeta recurrentis *aldolase*

(*a*) Metal ions alone. Tube contents were as given in Table 1; additions contained 1 μmole of metal salt. Extract was pre-incubated at 37° with metal ion for 10 min. before addition of substrate. Reaction was allowed to proceed for 15 min. at pH 8·5. Control contained no added metal

Metal	Activity (μg. of alkali-labile phosphorus/hr./mg. of protein)
Control	545
Fe^{2+}	272
Co^{2+}	1080
Mg^{2+}	539
Mn^{2+}	408
Zn^{2+}	618

(*b*) EDTA plus metal ions. 1 μmole of EDTA was added to the tubes first, then cell-free extract and then metal ion (2 μmoles). Incubated at 37° for 10 min. Control contained no added metal or EDTA

Metal	Activity (μg. of alkali-labile phosphorus/hr./mg. of protein)
Control	543
Fe^{2+}	403
Co^{2+}	1350
Mg^{2+}	272
Mn^{2+}	260
Zn^{2+}	1075
No metal	270

(*c*) Metal ions plus cysteine. 2 μmoles of metal were added to the tubes first, followed by cell-free extract. Tubes were incubated for 10 min. at 37°. Hydrogen was bubbled through the reaction mixture before addition of cysteine (6 μmoles). Control contained no added metal or cysteine

Metal	Activity (μg. of alkali-labile phosphorus/hr./mg. of protein)
Control	530
Fe^{2+}	750
Co^{2+}	1955
Mg^{2+}	1575
Mn^{2+}	1140
Zn^{2+}	1215
No metal	1760

Table 3. *Dialysis of cell-free extracts of* Spirochaeta recurrentis *in the presence and absence of* Co^{2+} *and* Zn^{2+} *ions*

Tube contents, in duplicate, consisted of 3 ml. of cell-free extract diluted five times with ice-cold 0·9% NaCl, pre-incubated for 10 min. at 37° in the presence or absence of metal ions (5 mM). Solutions were then placed in dialysis sacs each immersed in 50 ml. of 0·9% NaCl, and rocked mechanically for 48 hr. at 2°. The 0·9% NaCl was changed once during this time. Undialysed controls were pre-incubated for the same length of time and also kept at 2°. Activity assays were then carried out.

Additions before dialysis	Activity (μg. of alkali-labile phosphorus/hr./mg. of protein)	
	Undialysed	Dialysed
None	392	473
Co^{2+}	203	338
Zn^{2+}	470	470

for crystalline enolase. Hydrogen gas was then bubbled through the reaction mixture and three times as much cysteine as metal was added to activate the sulphydryl groups of the enzyme. It was assumed that in this manner there would be sufficient of the metal ion adjacent to the enzyme, and ample cysteine available to provide activity even after chelation had taken place.

The results (Table 2c) do in fact show that in the presence of cysteine Co^{2+} ions give maximal activity and, making allowance, for controls without added metal and with cysteine alone, that Fe^{2+}, Mn^{2+} and Mg^{2+} ions inhibit in that order. Anomalously, zinc appears to inhibit although, as shown earlier, it can apparently activate the enzyme.

(*d*) Effect of dialysis on aldolase activity. Owing to the loss of activity exhibited by spirochaetal extracts on storage in the cold, a property also of clostridial extracts noticed by Bard & Gunsalus (1950), no clear-cut conclusions on the role of metal ions could be drawn from the results of dialysis experiments. It was noticed, however, that dialysed extracts appeared to retain their aldolase activity longer than undialysed ones. Pre-incubation in the presence of Co^{2+} ions resulted in a more rapid loss of activity on storage or dialysis than did pretreatment with Zn^{2+} ions. Lastly, in some way zinc appeared to preserve the activity of the undialysed extract. These results are summarized in Table 3.

DISCUSSION

It should be emphasized that all the results reported here were obtained by use of the chemical triose-trapping method of aldolase assay, which is not prone to the interfering side reactions involved in the use of coupled optical systems with crude

Table 4. *Properties of some aldolases from different sources*

—, Indicates that no data were given.

Source	Assay system*	pH optimum	$10^3 K_m$ (M)	Activators	Inhibitors	Reference
Clostridium perfringens	T	7·5	1	Fe^{2+}, Co^{2+} cysteine	αα′-Dipyridyl, *o*-phenanthroline, pyrophosphate	Bard & Gunsalus (1950)
Clostridium butyricum	T	7·6	1	Fe^{2+}, Co^{2+} cysteine	Fe^{3+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, αα′-dipyridyl, *o*-phenanthroline, pyrophosphate	Gavard (1954)
Pasteurella pestis	T	8·6	1·3†	Co^{2+} cysteine	Fe^{2+}, Zn^{2+}, Mn^{2+}, *o*-phenanthroline, EDTA	Srikantan, Krishna Murti & Shrivastava (1958)
Brucella suis	T	7·0–7·5	—	Fe^{2+} cysteine	αα′-Dipyridyl, *o*-phenanthroline	Gary, Kupferberg & Graf (1955)
BCG	T	—	—	Zn^{2+}	Co^{2+}, Ca^{2+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, cysteine, phosphate	Su & Ling (1956)
Aspergillus niger	O	7·5	1·5	Zn^{2+}	Cysteine, EDTA, pyrophosphate, *o*-phenanthroline, αα′-dipyridyl	Jagannathan, Singh & Damodaran (1956)
Yeast	O	—	—	Zn^{2+}, Fe^{2+}, Co^{2+}	Cysteine, αα′-dipyridyl, pyrophosphate	Warburg & Christian (1942)
Spirochaeta recurrentis	T	8·75	1·4	Co^{2+}, Zn^{2+}, cysteine	Fe^{2+}, Mn^{2+}, αα′-dipyridyl, 8-hydroxyquinoline, EDTA	Present investigation
Trypanosoma cruzi	T	7·3	1·2	—	Cu^{2+}, pyrophosphate, 8-hydroxyquinoline	Baernstein & Rees (1952)
Trichomonas vaginalis	T	7	1	Fe^{2+}, Co^{2+}	Cu^{2+}, Zn^{2+}, αα′-dipyridyl, EDTA	Baernstein (1954)
Muscle (rabbit)	T	9	1	No metal activation	Cu^{2+}, Ag^{+}, I_2	Herbert, Gordon, Subrahmanyan & Green (1940)

* T, trapping trioses; O, optical.

† Derived from published data.

extracts. With pure enzyme preparations the optical assay is the method of choice, and by its means Baranowski & Niederland (1949) found a pH optimum of 6·7–7·5 for crystalline rabbit-muscle aldolase. These authors supposed that trapping by chemical means becomes incomplete at pH values below 9, thus simulating a decrease in the rate of aldolase activity. This may be the reason for the high value of pH 9 quoted by Herbert, Gordon, Subrahmanyan & Green (1940) and by Peanaskey & Lardy (1958), who used cyanide to trap the trioses formed in the muscle- and liver-aldolase reaction. There may be reason to believe that this is the case with hydrazine also, for *S. recurrentis* aldolase activity falls off sharply on either side of pH 8·5–9·0. However, Bard & Gunsalus (1950), Baernstein & Rees (1952), Baernstein (1954) and Srikantan, Krishna Murti & Shrivastava (1958) all used hydrazine as a trapping agent and were able to show different pH-dependent behaviour for their enzyme preparations. The general shape of the curve for *S. recurrentis* aldolase most closely resembles that of Bard & Gunsalus for *Clostridium perfringens*. Moreover, the optimum reported in the previous paper for the diphosphopyridine nucleotide-reducing system with FDP as substrate and catalysed by spirochaetal extract also lay between pH 8 and 9.

There is a close correlation, too, between the K_m 1·4 mM for FDP in the aldolase-catalysed reaction and the optimum FDP concentration in the diphosphopyridine nucleotide-reducing system. It seems probable therefore that the rate-limiting reaction in the system is the aldolase-catalysed splitting of FDP. The K_m value obtained here is close to the value of 1·3 mM derived from the experimental data of Srikantan *et al.* (1958) for the partially purified aldolase of *Pasteurella pestis*, examined under similar test conditions. Values for purified and unpurified aldolase from different sources have been obtained by various workers and a list of some of these is included in Table 4. Most of the K_m figures quoted are about 1 mM, depending upon the conditions of assay. One of the difficulties in drawing comparisons of this kind lies in variation in experimental parameters affecting the Michaelis constant (Dixon & Webb, 1958), so that often there is no common ground on which to compare differences.

The most striking character affecting the activity of aldolase is perhaps its behaviour towards metal ions. Depending upon its source, the enzyme either does or does not require the participation of metal ions for maximal activity. Herbert *et al.* (1940), for instance, showed that traces of heavy metals inactivated the crystalline enzyme from rabbit muscle. By contrast, the enzyme from yeast first isolated by Warburg & Christian (1942) does not show full activity unless previously incubated in the presence of either bivalent zinc, cobalt, iron or copper ions. In this respect the muscle enzyme appears to stand alone, because aldolases from *Clostridium perfringens* (Bard & Gunsalus, 1950), *C. butyricum* (Gavard, 1954), *Aspergillus niger* (Jagannathan *et al.* 1956) and *Pasteurella pestis* (Srikantan *et al.* 1958) also show bivalent-metal activation; the clostridial and *Pasteurella* aldolases are, in addition, cysteine-activated (see Table 4).

In the experiments described above both cobalt and zinc were found to exert an activating effect upon the spirochaetal enzyme. With cysteine, however, zinc appeared to be inhibitory. This behaviour might be explained if it is tentatively assumed that cobalt is the natural activator of *S. recurrentis* aldolase, and that zinc is able to replace it under certain conditions. Both zinc and cobalt are readily chelated by cysteine (Albert, 1952), the association constant for zinc being somewhat higher than that for the other ion. Consequently if zinc were not so firmly bound to the enzyme it might be removed more easily by the cysteine. In addition, experiments showed that zinc was able to relieve the inhibition produced by EDTA, but not to such an extent as cobalt. If the above assumption is correct therefore, it is probable, because of the close similarity of Co^{2+}– and Zn^{2+}–EDTA-complex association constants (Martell & Calvin, 1952) that EDTA also is able to remove some of the zinc from the protein. By reason of its close similarity to cobalt in ionic radius and electronegativity (Wold & Ballou, 1957) it seems hardly surprising that zinc can substitute for cobalt in the activation of *S. recurrentis* aldolase. In fact the data given in Table 4 show that a proportion of aldolases are activated by these metals.

The significance of the finding that metal ions are themselves an indispensable feature of the activity of the spirochaete enzyme is emphasized by recent work on the classification of plant and animal aldolases on the basis of their inhibition or non-inhibition with EDTA (Bertrand & De Wolf, 1958). These authors' results show that greatest metal-ion dependence appears to exist among the aldolases of the lower forms of life of both plant and animal kingdoms. This being the case, it is felt that closer attention to purification of the aldolase of *S. recurrentis*, and the enzyme from other sources, may reveal the presence of a single-enzyme single-metal relationship similar to that existing amongst the zinc metallo-dehydrogenases.

SUMMARY

1. Some of the properties of a diphosphofructo-aldolase in cell-free extracts of *Spirochaeta recurrentis* have been studied.

2. By the hydrazine triose-trapping method, a pH optimum of 8·75 and an approximate K_m 1·3 mM have been demonstrated. These values are similar to those derived for the enzyme from other sources.

3. The enzyme is activated by Co^{2+} and Zn^{2+} ions, cysteine and reduced glutathione. Fe^{2+}, Mn^{2+} and Mg^{2+} ions either inhibit or have no effect. Ethylenediaminetetra-acetate, 8-hydroxyquinoline, $\alpha\alpha'$-dipyridyl and *p*-chloromercuribenzoate inhibit aldolase activity. On the basis of these findings the spirochaetal enzyme is classed as a sulphydryl-group-containing metalloaldolase.

4. The extent of metal-ion activation of different aldolases is briefly discussed.

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REFERENCES

Albert, A. (1952). *Biochem. J.* **50**, 690.
Baernstein, H. D. (1954). *Fed. Proc.* **13**, 177.
Baernstein, H. D. & Rees, C. W. (1952). *Exp. Parasit.* **1**, 215.
Baranowski, T. & Niederland, T. R. (1949). *J. biol. Chem.* **180**, 543.
Bard, R. C. & Gunsalus, I. C. (1950). *J. Bact.* **59**, 387.
Bertrand, D. & De Wolf, A. (1958). *C.R. Acad. Sci., Paris*, **247**, 2213.
Cori, G. T., Slein, M. W. & Cori, C. F. (1948). *J. biol. Chem.* **173**, 605.
Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 151. London: Longmans Green and Co. Ltd.
Gary, N. D., Kupferberg, L. L. & Graf, L. H. (1955). *J. Bact.* **69**, 478.
Gavard, R. (1954). *C.R. Acad. Sci., Paris*, **238**, 1620.
Gilbert, J. B., Otey, M. C. & Price, V. E. (1951). *J. biol. Chem.* **190**, 377.
Gunsalus, I. C., Horecker, B. L. & Wood, W. A. (1955). *Bact. Rev.* **19**, 79.
Hellerman, L., Chinard, F. P. & Deitz, V. R. (1943). *J. biol. Chem.* **147**, 443.
Herbert, D., Gordon, H., Subrahmanyan, V. & Green, D. E. (1940). *Biochem. J.* **34**, 1108.
Jagannathan, V., Singh, K. & Damodaran, M. (1956). *Biochem. J.* **63**, 94.
Klotz, I. M. & Hughes, T. R. (1956). *Meth. biochem. Anal.* **3**, 265.
Krishna Murti, C. R. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 4–102.
Leuthardt, F. & Wolf, H. P. (1954). *Helv. chim. acta*, **37**, 1734.
Madsen, N. B. & Cori, C. F. (1956). *J. biol. Chem.* **223**, 1055.
Malmström, B. G. (1954). *Arch. Biochem. Biophys.* **49**, 335.
Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*. New York: Prentice-Hall Inc.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Neuberg, C. & Lustig, H. (1942). *J. Amer. chem. Soc.* **64**, 2722.
Neuberg, C. & Sabatay, S. (1925). *Biochem. Z.* **161**, 240.
Peanaskey, R. J. & Lardy, H. A. (1958). *J. biol. Chem.* **233**, 365.
Rabin, B. R. (1955). *Disc. Faraday Soc.* **20**, 260.
Rose, I. A. & Rieder, S. V. (1958). *J. biol. Chem.* **231**, 315.
Sibley, J. A. & Lehninger, A. L. (1949). *J. biol. Chem.* **177**, 859.
Smith, P. J. C. (1960). *Biochem. J.* **76**, 500.
Srikantan, T. N., Krishna Murti, C. R. & Shrivastava, D. L. (1958). *Indian J. med. Res.* **46**, 1.
Stumpf, P. K. (1954). In *Chemical Pathways of Metabolism*, vol. 1, p. 68. Ed. by Greenberg, D. M. New York: Academic Press Inc.
Su, T-C. & Ling, K. H. (1956). *J. Formosan med. Ass.* **55**, 216.
Taylor, J. F. (1955). In *Methods in Enzymology*, vol. 1, p. 310. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **311**, 209.
Warburg, O. & Christian, W. (1943). *Biochem. Z.* **314**, 149.
Williams, R. J. P. (1953). *Biol. Rev.* **28**, 381.
Wold, F. & Ballou, C. E. (1957). *J. biol. Chem.* **227**, 313.

Biochem. J. (1960) **76**, 514

Carbohydrate Metabolism in *Spirochaeta recurrentis*

4. SOME PROPERTIES OF HEXOKINASE AND LACTIC DEHYDROGENASE IN SPIROCHAETES*

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The properties of enzymes occurring initially or terminally in a particular metabolic sequence often provide useful information about the rate-controlling processes which govern their activities in the intact organism (see, for example, Krebs, 1957; Krimsky, 1959; Holzer, 1959). Particular susceptibility to inhibition and hormonal influence is shown by hexokinase, the enzyme catalysing the

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Table 1. *Effect of various additions on the hexokinase of* Spirochaeta recurrentis *under standard-assay conditions*

Reaction mixture contained: 0·1 ml. of KH_2PO_4, 10 μmoles; 0·1 ml. of EDTA, 8 μmoles; 0·5 ml. of ATP, 20 μmoles; 0·3 ml. of $MgSO_4$, 45 μmoles; 0·2 ml. of glucose, 160 μmoles; 2·0 ml. of homogenate, approx. 10 mg./ml.; 0·1 ml. additions. Water to 4 ml. Reaction was started by addition of substrate. Temp. 35°. pH was maintained at 7·5 by addition of standard alkali (0·1 N-NaOH).

Additions	Final concn.	Activity (μmoles of glucose transformed/hr.)
None	—	30·6
Glucose 6-phosphate dehydrogenase + TPN^+	0·1 mg./4 ml. 0·5 mg./4 ml.	34·2
Fluoride	25 mM	0·4
Fluoride	12 mM	13·6
Fluoride	6 mM	35·5

primary phosphorylation of glucose by adenosine triphosphate (Lepage, 1950; Stadie, 1954). Similarly, in those organisms producing lactic acid from glucose, diphosphopyridine nucleotide-dependent lactic dehydrogenase may function as a metabolic regulator to an extent measurable by conventional methods of enzyme-kinetic analysis (Neilands, 1952; Nygaard, 1956). As both hexokinase and lactic dehydrogenase have been shown to be functional in *Spirochaeta recurrentis* (Smith, 1960), some of the properties of these two enzymes are reported, with special reference to their possible role in the carbohydrate metabolism of this parasitic spirochaete.

EXPERIMENTAL

Methods

Methods for the preparation of spirochaetal 'homogenates' (disintegrated cells) and cell-free extracts have been given in a preceding paper (Smith, 1960).

Abbreviations. DPN^+, DPNH, oxidized and reduced forms of diphosphopyridine nucleotide; ADP, ATP, adenosine di- and tri-phosphate.

Hexokinase assay. After the photometric-indicator assays of Wajzer (1949) and of Crane & Sols (1955), and the methods of other authors (Kerly & Leaback, 1957; Slein, Cori & Cori, 1950), had been used initially without success, the assay finally adopted, because of its sensitivity and relative simplicity, was the micro-titrimetric constant-pH method (Schwartz & Meyers, 1958), which has already been described (Smith, 1960). Rate of formation of acid in micro-equivalents at neutral pH, after blank corrections, was taken to be equal to the rate of phosphorylation of glucose, which was expressed as μmoles of substrate consumed/hr./mg. dry wt. of homogenate.

Lactic-dehydrogenase assay. Routine assay of the pure crystalline commercial preparation, and of the activity of the enzyme in spirochaetal extracts, was performed in the manner described by Smith (1960).

The reaction in the reverse direction, with lactic acid as substrate, was carried out after the manner of Neilands (1955). In this, excess of lactic acid and of DPN^+ were used in the presence of veronal–acetate buffer (0·14 M) (Michaelis, 1931) at pH 7·75. The glycine–NaOH buffer, pH 10, recommended by Neilands, was not used for reasons given below.

Materials

General reagents were of AnalaR-reagent grade where possible and further purification was not attempted unless necessary. Pure crystalline sugars and D(+)-glucosamine hydrochloride were purchased from British Drug Houses Ltd. (Poole, Dorset). *N*-Acetyl-D-glucosamine was from L. Light and Co. Ltd. Sources of enzymes, coenzymes, sugar phosphates and sodium pyruvate have been given previously Smith (1960). Pure DL-lactic acid was prepared from commercial lactic acid (British Drug Houses Ltd.) via the zinc salt. Treatment of the zinc lactate with Zeo-Karb 225/H (The Permutit Co. Ltd., London) gave the free acid, from which sodium DL-lactate solution was obtained by neutralization with standard alkali. This is similar to the method used by Hakala, Glaid & Schwert (1956).

RESULTS

Hexokinase

Generally, activity was proportional to the quantity of spirochaetal homogenate added, and amounted to between 0·3 and 0·4 mg. of glucose consumed/hr./mg. dry wt. This is less than the glucose utilized by the whole spirochaetes (0·5–0·75 mg./hr./mg. dry wt.). Results varied from batch to batch, however, and, as activity fell rapidly after thawing, repeated experiments on the same homogenate were not always possible. Therefore experiments described here were of necessity limited by the labile nature of the homogenates themselves.

Effect of added zwischenferment and triphosphopyridine nucleotide. As inhibition by D-glucose 6-phosphate has been shown to occur with the hexokinase of rat-brain extracts by Weil-Malherbe & Bone (1951) and by Crane & Sols (1954), this product of the reaction was removed *in situ* by adding zwischenferment (glucose 6-phosphate dehydrogenase) and its coenzyme TPN^+ to the reaction mixture. Table 1 shows that little increase in the activity was obtained, and the slowness of

the catalysis by spirochaetal hexokinase was unlikely therefore to be due to accumulation of glucose 6-phosphate. This experiment obviated also the chance that phosphofructokinase was contributing to acid production.

No glucose utilization occurred in the absence of ATP, but addition of larger amounts of ATP to the system did not increase the rate.

Effect of fluoride. Fluoride was added to the reaction mixture in some experiments to inhibit any adenosine triphosphatase present, and because Ochoa (1941) found difficulty in demonstrating the hexokinase of brain extracts in its absence. The precautions to be taken in using F^- ions in the hexokinase assay, however, where magnesium is required for optimum activity, have been emphasized by Melchior & Melchior (1956).

Addition of 100μmoles of sodium fluoride to the reaction mixture before the start inhibited the activity completely. But, if addition was made after pre-incubation of the homogenate with ATP and Mg^{2+} ions, the inhibition was not so marked. However, in no instance did fluoride appear to enhance the activity of the spirochaetal enzyme (Table 1).

Substrate specificity. Table 2 shows the hexokinase activity of the homogenates in the presence of various substrates. By far the greatest activity was shown with glucose, and then, in descending order, with mannose, sorbose, fructose and galactose. Even when allowance was made for its acid-binding power D(+)-glucosamine was not utilized at all, whereas it has been shown to serve as a substrate for the hexokinases from yeast (Brown, 1951; Grant & Long, 1952) and from brain (Sols & Crane, 1954). In fact, as shown below, this amino sugar apparently inhibited the utilization of glucose by spirochaetal homogenates.

Inhibition of hexokinase. Inhibition by glucosamine was found to be proportional to the amount added, as Table 3 shows. An extensive study of the inhibition of hexokinase by synthetic *N*-acyl derivatives of D-glucosamine (Maley & Lardy 1955) revealed that *N*-acetyl-D-glucosamine was highly specific, and inhibitory in a concentration as low as 1μM. The results obtained with this synthetic-substrate analogue showed that it, too, like the unsubstituted glucosamine, inhibited the hexokinase activity of *S. recurrentis* homogenates. To obtain maximal inhibition, however, it was necessary to add each inhibitor before the glucose, which suggests that, as with brain hexokinase (Sols & Crane, 1954), they compete with glucose for the enzyme surface.

Table 2. *Substrate specificity of* Spirochaeta recurrentis *hexokinase*

Reaction mixtures contained the same components as those described in Table 1, without extra additions. Equimolar amounts (160μmoles/4 ml. of reaction mixture) of the substrate indicated were added, in 0·2 ml., to start the reaction. Temp. 35°. pH was maintained at 7·5.

Substrate	Activity (μmoles/hr./mg. dry wt.)	Activity (as percentage of glucose activity)
D(+)-Glucose	1·32	100
D(−)-Fructose	0·38	29
D(+)-Galactose	0·03	2
D(+)-Mannose	1·25	95
L(−)-Sorbose	0·61	46
D(+)-Glucosamine	0·00	0

Lactic dehydrogenase

Effect of extract concentration. Comparatively small quantities of cell-free extract brought about rapid oxidation of DPNH when pyruvate was used as substrate. The increase in velocity followed linearly the increase in the amount of extract added to the cuvette (Fig. 1).

Effect of pH. A comparison was made of the pH optima of purified muscle lactic dehydrogenase and that contained in *S. recurrentis* extracts. The activities of both enzymes were adjusted by dilution until nearly equal. When tests were conducted in veronal–acetate buffer at 20° the optimum for both muscle and spirochaetal enzymes was at pH 7·0, and the curves were closely similar in shape (Fig. 2).

Table 3. *Inhibition of glucose utilization in the* Spirochaeta recurrentis *hexokinase reaction*

Reaction mixtures contained the same components as those described in Table 1, except that additions were made before starting the reaction with glucose. Temp. 35°. pH was maintained at 7·5.

Inhibitor	Concn. (mM)	Activity (μmoles/hr./mg. dry wt.)	Inhibition (%)
None	—	2·41	0
D(+)-Glucosamine	10	1·25	48
D(+)-Glucosamine	40	0·00	100
N-Acetyl-D-glucosamine	0·01	1·75	27
N-Acetyl-D-glucosamine	1·0	0·68	72
N-Acetyl-D-glucosamine	10	0·34	86

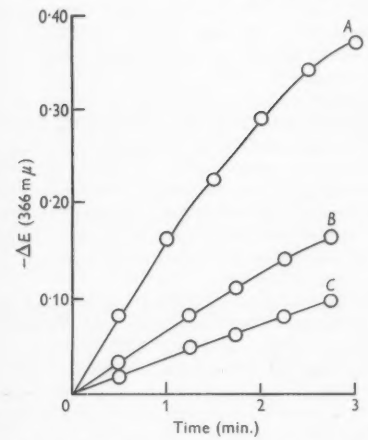

Fig. 1. Effect of extract concentration on lactic-dehydrogenase activity. Cuvettes contained: 2·65 ml. of triethanolamine buffer (0·05 M), pH 7·5; 0·05 ml. of DPNH, 10 mg./ml.; 0·2 ml. of sodium pyruvate, 7 μmoles; cell-free extract containing, *A*, 220 μg., *B*, 110 μg., *C*, 70 μg. of protein. Control, without substrate, tested for absence of DPNH-oxidase activity. Blank contained water. Reaction was started by addition of extract. Temp. 20°.

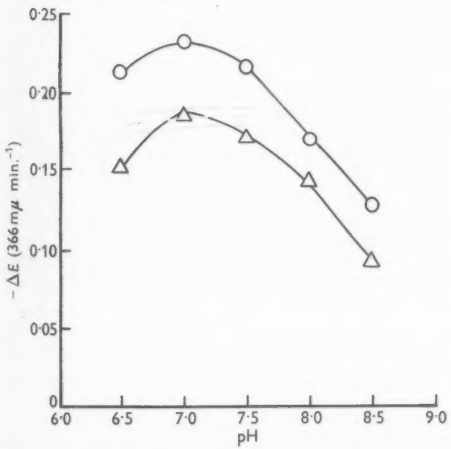

Fig. 2. Effect of pH on lactic-dehydrogenase activity. Cuvette contents: 2·7 ml. of veronal–acetate buffer (0·14 M), of pH shown above; 0·05 ml. of DPNH, 10 mg./ml.; 0·2 ml. of sodium pyruvate, 7 μmoles; 0·05 ml. of extract, or pure lactic dehydrogenase suitably diluted, was added last to start the reaction. The blank contained water. Temp. 20°. ○, Spirochaetal cell-free extract, 70 μg. of protein; △, crystalline muscle lactic dehydrogenase, 0·5 μg. of protein.

Effect of pyruvate concentration. Fig. 3 illustrates how the enzymic activity of the extracts responded to an increase in the substrate concentration. Activity reached a maximum at about 2·5 mM-pyruvate and then appeared to fall off slightly at higher concentration. The concentration of pyruvate corresponding to half-maximal velocity, the K_m of Michaelis–Menten kinetics (Michaelis & Menten, 1913), is approx. 0·3 mM. Owing to the unpurified nature of the enzyme being studied it was not considered justifiable to apply the more accurate Lineweaver & Burk (1934) treatment.

Reverse reaction, lactate oxidation. The glycine–NaOH buffer system used by Neilands (1955) at pH 10 gave a very low level of activity with spirochaete extract. After several buffers at different pH values had been tried, veronal–acetate buffer, pH 7·75, was used successfully. The curve shown in Fig. 4 is typical of the results obtained, with DL-lactate as substrate. The amount of DPN^+ reduced during the oxidation of the lactate is comparatively small because the reaction rapidly reaches equilibrium.

Effect of lactate concentration. Kubowitz & Ott (1943) found that D(−)-lactate would not act as a substrate for muscle or tumour lactic dehydrogenase; nor will it interfere with the oxidation of the L(+)-isomer (Neilands, 1952). The majority of the lactic dehydrogenases from animal sources are stereospecific for L-lactic acid (Schlenk, 1951), whereas those of micro-organisms produce D-, L-

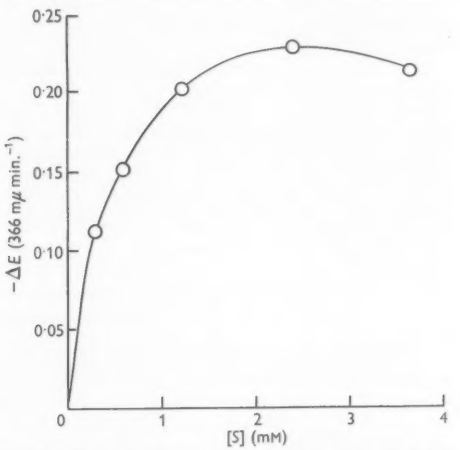

Fig. 3. Effect of pyruvate concentration on lactic-dehydrogenase activity. Cuvette contents were as described for Fig. 1, except that increasing concentrations of pyruvate were used, as indicated. Extract contained 35 μg. of protein in 0·05 ml. used in tests. The blank contained water. Temp. 20°.

or DL-forms (Katagiri & Kitahara, 1937). It seemed highly probable that the spirochaetal enzyme also exhibits L-lactate specificity, because of the possibility that disposal of this metabolic end product of the parasite's metabolism takes place via the respiratory pathways of the host. Therefore it was tentatively assumed that the D(−)-lactate present in the substrate solution as prepared from DL-lactic acid would not interfere in the reaction catalysed by the enzyme from *S. recurrentis*, and concentration of substrate was calculated on the basis of the L(+)-lactate it contained, that is, half the amount of DL-lactate.

Examination of Fig. 5 reveals that maximal velocity is attained when the concentration of L(+)-lactate is 42 mM, which gives K_m about 10 mM with respect to L(+)-lactate. By comparing the K_m values for both forward and backward reactions it is apparent that the affinity of the enzyme for lactate is about one-thirtieth of that for pyruvate.

Equilibrium constant. Results obtained from equilibrium assays of the spirochaetal lactic dehydrogenase with lactate as substrate were used to calculate two approximate constants for the reaction equilibrium. No account was taken of the pyruvate kinase-catalysed phosphorylation of pyruvate to phosphoenolpyruvate, since the kinase was shown earlier to be relatively inactive in the absence of added ATP.

The concentration of DPNH was calculated from the extinction constants given by Horecker & Kornberg (1948), and the hydrogen term was omitted and affinity constants were assumed to be unity. The 'apparent' equilibrium constant ($K_{app.}$), derived from the figures quoted in Table 4, is $6{\cdot}1 \times 10^{-5}$.

Fig. 4. Lactate oxidation catalysed by *S. recurrentis* extract. Cuvette contained: 2·75 ml. of veronal–acetate buffer (0·14 M), pH 7·75; 0·1 ml. of DPN, 20 mg./ml.; 0·1 ml. of sodium DL-lactate, 9 mg.; 0·05 ml. of cell-free extract, 100 μg. of protein. The blank contained water in place of substrate. Temp. 20°.

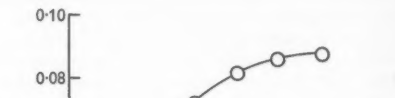

Fig. 5. Effect of L(+)-lactate concentration on lactic-dehydrogenase activity. Cuvette contents were as described in Fig. 4, except that the concentration of lactate shown above, calculated on the basis of L(+)-lactate content, was used. Temp. 20°.

Table 4. *Equilibrium constants of the lactic-dehydrogenase reaction catalysed by* Spirochaeta recurrentis *extracts*

Measurement was made in veronal–acetate buffer (0·14 M), pH 7·75, in a 1 cm. cell; 3 ml. total volume at 20°. I and II refer to two experiments with different concentrations of lactic acid.

	Initial concn. (M)		Final concn. (M)	
Component	I	II	I	II
L(+)-Lactic acid	$13{\cdot}5 \times 10^{-3}$	$6{\cdot}75 \times 10^{-3}$	$13{\cdot}4 \times 10^{-3}$	$6{\cdot}65 \times 10^{-3}$
DPN^+	$9{\cdot}5 \times 10^{-4}$	$9{\cdot}5 \times 10^{-4}$	$9{\cdot}2 \times 10^{-4}$	$9{\cdot}3 \times 10^{-4}$
Pyruvic acid	0	0	$29{\cdot}3 \times 10^{-6}$	$17{\cdot}9 \times 10^{-6}$
DPNH	0	0	$29{\cdot}3 \times 10^{-6}$	$17{\cdot}9 \times 10^{-6}$
		$K_{app.}$	$7{\cdot}0 \times 10^{-5}$	$5{\cdot}2 \times 10^{-5}$

K average $= 6{\cdot}1 \times 10^{-5}$.

Owing to the difficulty in obtaining sufficient experimental material no attempt was made to purify any of the enzymes of *S. recurrentis*, so that these values are approximate. Because of its relative stability on storage in the freeze-dried organisms, however, it is felt that purification of spirochaetal lactic dehydrogenase is a distinct possibility.

DISCUSSION

Attention has been drawn previously (Smith, 1960) to the finding that the hexokinase of *S. recurrentis* is associated with the particulate fraction of the organism. This, together with the unsuitability of hexose phosphates as substrates for the intact spirochaetes, points to the possibility that this hexokinase may be an ectoenzyme found on the cell surface, as is supposed to be the case with mouse ascitic-tumour cells (Kazakova, 1958). Soluble and particulate hexokinases have been known for some time (Slein *et al.* 1950; Crane & Sols, 1953), but they are not usually as difficult to demonstrate as was the spirochaetal enzyme. In fact, this difficulty, by comparison with the ease with which aldolase and lactic dehydrogenase were demonstrated, may be a manifestation of an inhibition significant in the control of *S. recurrentis* glycolysis.

The hexokinase reaction is known to be very sensitive to quite low concentrations of ADP (Lepage, 1950), the process of inhibition constituting a 'pacemaker' system, according to Krebs (1957). Also, kinetic studies by Beck (1955) on the rates of anaerobic glycolysis in human leucocytes have shown that although the functional capacity, $V_{max.}$, of an individual enzyme may be demonstrably high, its actual performance in the metabolic chain may be low or limiting. These facts suggest therefore that in *S. recurrentis* the apparent slowness of the hexokinase reaction may reflect a mechanism whereby the speed of passage of the phosphorylated glycolytic intermediates through the Embden–Meyerhof pathway is regulated to make most economical use of the available substrate. Some measure of support is provided for this view by recent work of Randle & Smith (1958), showing that entry of glucose into muscle cells is normally the rate-limiting step in its metabolism.

Whether fructose is genuinely unsuitable as a substrate, or whether its measurably slower rate of phosphorylation by both whole and disintegrated spirochaetes is due to its enzymically inactive pyranose form in solution (Gottschalk, 1947), must await results of further inquiry.

From the limited investigation made of its properties, the lactic dehydrogenase of *S. recurrentis* must resemble its mammalian counterpart. The inhibitory effect of pyruvate at high concentrations was first noticed by Kubowitz & Ott (1943) for their enzyme from rat muscle. But the spirochaetal enzyme K_m of 0·3 mM with pyruvate does not resemble the value for the muscle enzyme, which is variously quoted at 90μM (Kubowitz & Ott, 1943), 52μM (Meister, 1950), of the order of 10μM (Neilands, 1952) and 54μM (Kun, 1957). Constants for pyruvate six to twelve times as high as those for the mammalian-muscle enzyme have been found, however, for lactic dehydrogenase of *Schistosoma mansoni* by Mansour & Bueding (1954). Though no justification for far-reaching conclusions, these quantitative data on the *S. recurrentis* lactic dehydrogenase were thought to be of some value in assessing the relative importance of the terminal step in glucose catabolism by the spirochaete. For similar reasons, and to determine the direction favoured, investigation of the reverse reaction from lactate to pyruvate was carried out.

Comparison of the K_m for both forward and backward spirochaete lactic dehydrogenase-catalysed reactions showed that affinity for lactate was one-thirtieth of that for pyruvate. Neilands (1952) has shown that the K_m for heart-muscle lactic dehydrogenase at pH 7, 8, 9 and 10 was about 10μM for DPN^+ and pyruvate. However, over the same pH range the K_m for L(+)-lactate was greater than 1 mM, that is, an affinity of about one-hundredth. The mean value for the equilibrium constant of the *S. recurrentis* enzyme is almost identical with the figure of $6{\cdot}0 \times 10^{-5}$ obtained for the rat and tumour enzyme by Kubowitz & Ott (1943). Both values are small, which means that the standard free-energy change (ΔF) has a large positive value and the reaction will not proceed far in the reverse direction spontaneously.

A possibly more important controlling influence in the lactic-dehydrogenase reaction can be presumed from its requirement for DPNH as a coenzyme. Any process tending to deplete sources of the pyridine nucleotide would result in an inhibition of the lactic-dehydrogenase-catalysed reaction. The gradual accumulation of pyruvic acid during the course of spirochaetal fermentation, far from being indicative of a steady-state equilibrium, could, indeed, reflect a metabolic inadequacy in the form of insufficient reduced coenzyme. Glock & McLean (1955) found that in all the animal tissues investigated the DPN^+ was present mainly in the oxidized form, whereas Holzer, Schultz & Lynen (1956) found this to be the case in living yeast cells. Under certain conditions therefore terminal conversion of pyruvate into lactate in *S. recurrentis* might be governed by the availability of DPNH.

SUMMARY

1. The particulate hexokinase of *Spirochaeta recurrentis* shows maximum specificity towards

D-glucose and D-mannose. L-Sorbose and D-fructose may substitute for them to a less-marked extent. The enzyme is inactive with D-galactose and inhibited, it is assumed competitively, by D-glucosamine and *N*-acetyl-D-glucosamine. Fluoride does not enhance its activity.

2. Some of the properties also of lactic dehydrogenase have been studied in cell-free extracts of the organism. Under similar spectrophotometric-assay conditions both mammalian-muscle and *S. recurrentis* lactic dehydrogenases were optimally active at pH 7·0.

3. The conversion of pyruvate into lactate was investigated in the forward and backward direction. K_m values of 0·3 mM and 10 mM were obtained for pyruvate and L-lactate respectively. The approximate equilibrium constant for the reaction was 6×10^{-5}.

4. The significance of these findings and possible functions of the two enzymes as regulators of the carbohydrate metabolism of *S. recurrentis* are discussed.

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REFERENCES

Beck, W. S. (1955). *J. biol. Chem.* **216**, 333.
Brown, D. H. (1951). *Biochem. biophys. Acta*, **7**, 487.
Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.
Crane, R. K. & Sols, A. (1954). *J. biol. Chem.* **210**, 597.
Crane, R. K. & Sols, A. (1955). In *Methods in Enzymology*, vol. 1, p. 277. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Glock, G. E. & McLean, P. (1955). *Biochem. J.* **61**, 388.
Gottschalk, A. (1947). *Biochem. J.* **47**, 478.
Grant, P. T. & Long, C. (1952). *Biochem. J.* **50**, xx.
Hakala, M. T., Glaid, A. J. & Schwert, G. W. (1956). *J. biol. Chem.* **221**, 191.
Holzer, H. (1959). In *Ciba Foundation Symp., Regulation of cell metabolism*, p. 277. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
Holzer, H., Schultz, G. & Lynen, F. (1956). *Biochem. Z.* **328**, 252.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Katagiri, H. & Kitahara, K. (1937). *Biochem. J.* **31**, 909.
Kazakova, T. B. (1958). *Biokhimiya*, **23**, 620.
Kerly, M. & Leaback, D. H. (1957). *Biochem. J.* **67**, 245.
Krebs, H. A. (1957). *Endeavour*, **16**, 125.
Krimsky, I. (1959). *J. biol. Chem.* **234**, 232.
Kubowitz, F. & Ott, P. (1943). *Biochem. Z.* **314**, 94.
Kun, E. (1957). *Biochim. biophys. Acta*, **25**, 135.
Lepage, G. A. (1950). *Cancer Res.* **10**, 77.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Maley, F. & Lardy, H. A. (1955). *J. biol. Chem.* **214**, 765.
Mansour, T. E. & Bueding, E. (1954). *Brit. J. Pharmacol.* **9**, 459.
Meister, A. (1950). *J. biol. Chem.* **184**, 117.
Melchior, N. C. & Melchior, J. B. (1956). *Science*, **124**, 402.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Neilands, J. B. (1952). *J. biol. Chem.* **199**, 373.
Neilands, J. B. (1955). In *Methods in Enzymology*, vol. 1, p. 449. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Nygaard, A. P. (1956). *Acta chem. scand.* **10**, 408.
Ochoa, S. (1941). *J. biol. Chem.* **141**, 245.
Randle, P. J. & Smith, G. H. (1958). *Biochem. J.* **70**, 501.
Schlenk, F. (1951). In *The Enzymes*, vol. 2, part 1, p. 250. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Schwartz, M. & Meyers, T. C. (1958). *Analyt. Chem.* **30**, 1150.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Smith, P. J. C. (1960). *Biochem. J.* **76**, 500.
Sols, A. & Crane, R. K. (1954). *J. biol. Chem.* **210**, 581.
Stadie, W. C. (1954). *Physiol. Rev.* **34**, 52.
Wajzer, J. (1949). *C.R. Acad. Sci., Paris*, **229**, 1270.
Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. J.* **49**, 339.

Biochem. J. (1960) **76**, 520

The Biosynthesis of Chondroitin Sulphate

INCORPORATION OF SULPHATE INTO CHONDROITIN SULPHATE IN EMBRYONIC CHICK CARTILAGE

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D'Abramo & Lipmann (1957) have demonstrated that active sulphate, adenosine 3′-phosphate 5′-sulphatophosphate is formed by an extract of embryonic-chick cartilage. It was further shown that adenosine 3′-phosphate 5′-sulphatophosphate was responsible for sulphation of chondroitin sulphate synthesized *in vitro* when the extract was incubated with adenosine triphosphate and magnesium chloride. After the isolation of the unsulphated mucopolysaccharide chondroitin

from cornea (Davidson & Meyer, 1954), Meyer, Davidson, Linker & Hoffman (1956) suggested that this material was the direct precursor of chondroitin sulphate, i.e. that the molecule is polymerized before sulphation. Chondroitin, prepared by desulphation of chondroitin sulphate (Kantor & Schubert, 1957), did not act as a sulphate acceptor when incubated with the chick-cartilage extract (Adams, 1959*a*). Partially purified umbilical-cord hyaluronic acid, on the other hand, greatly stimulated chondroitin sulphate formation. Since hyaluronic acid does not occur as a sulphate ester, a chemical fractionation of an extract of umbilical cords in sodium chloride solution was undertaken and the fractions so obtained, along with other mucopolysaccharides, were tested for their effects on chondroitin sulphate formation in the chick-cartilage extract. A preliminary report of this work has already been published (Adams, 1959*b*).

METHODS

Preparation of chick-condyle enzyme

The enzyme used in this study was isolated from the condyles of the tibias and femora of 15-day-old chick embryos as described by D'Abramo & Lipmann (1957). A high-speed supernatant, prepared from 24 embryo condyles by extraction with 20 ml. of 0·01 M-phosphate in 0·9% NaCl, pH 7·4, was kept frozen at −40° in ampoules until required.

A suitable volume of a solution of each fraction to be tested for stimulating activity on chondroitin sulphate (CSA) biosynthesis was incubated with adenosine triphosphate (ATP), 5 μmoles; cysteine hydrochloride, 3 μmoles; $MgCl_2$, 6 μmoles; 2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer, pH 8·2, 60 μmoles; enzyme, 0·3 ml. of high-speed supernatant and radioactive tracer, usually 10–25 μc of carrier-free $Na_2{}^{35}SO_4$, based on the stated activity of the suppliers, The Radiochemical Centre, Amersham, Bucks. The total volume was 0·8 ml. (see Table 4).

Treatment of condyle enzyme with protamine. Protamine sulphate (Mann Research Laboratories) (500 mg.) was converted into the chloride by dissolving in water (10 ml.) and adding 10% (w/v) $BaCl_2$ solution until no further precipitate was obtained. The solution was then filtered, centrifuged to remove the small amount of suspended material, and the supernatant dialysed against several changes of 0·01 M-phosphate in 0·9% NaCl. To 10 ml. of high-speed extract of chick-embryo condyles kept at 4°, 1 ml. of the cold, dialysed protamine solution was added with stirring. After standing for 15 min. at 4°, the turbid mixture was centrifuged in the cold at 35 000 *g* for 20 min. A further 1·2 ml. of dialysed protamine solution was added before the clear supernatant, obtained by centrifuging, gave no further turbidity on addition of a drop of protamine solution. The precipitate was discarded and the supernatant used for the following experiment.

Incubation of protamine-treated enzyme with chondroitin sulphates A, B and C. It was found that addition of 0·3 ml. of a solution of CSA-A, containing 7 mg./ml., to a mixture of 0·3 ml. of the protamine-treated enzyme and 0·3 ml. of 0·2 M-tris buffer, pH 8·3 (Gomori, 1955) was sufficient to remove most of the free protamine; addition of more CSA-A solution to the supernatant obtained after centrifuging off the precipitate, failed to yield any further turbidity. Accordingly 0·3 ml. samples of protamine-treated enzyme were separately mixed at 4° with 0·4 ml. of solutions of the sodium salts of CSA-A, -B and -C, each containing 7 mg./ml. After standing for 15 min at 4°, the precipitate was removed by centrifuging and the supernatants were incubated at 37° with ATP etc., as shown in Table 6. After incubation, radioactive CSA was isolated in the normal manner.

Preparation of fractions from human umbilical cords

Extraction of cords. Full-term cords were collected, washed in water and stored under acetone. After washing with two further lots of acetone, the cords were air-dried, moistened with water and passed through a mechanical meat grinder. The ground material (3 kg. wet wt.) was then suspended in 0·9% NaCl (2 l.) stirred occasionally and left overnight. The viscous mass was filtered through layers of cheese cloth on a Büchner funnel and the residue in the cloth was then treated again with 0·9% NaCl (2 l.) and the reddish brown extracts were combined (2·2 l.).

Fractionation of extract. Ethanol (2·5 vol.) was added, with stirring, to the above extract. After standing overnight at 4°, the precipitated stringy material was collected by centrifuging, washed with ethanol–water (80:20, v/v) and extracted by stirring with 0·5 M-sodium acetate buffer, pH 5 (1 l.). The mixture was centrifuged and ethanol (1·5 vol.) added to the supernatant. The mixture was left at 4° overnight and the stringy precipitate was collected by centrifuging and washed with ethanol–water (75:25, v/v) ethanol–water (95:5, v/v) and ether. This material (5·2 g.) was dissolved in water (1 l.) and further fractionated as outlined in Table 1.

Fractionation of partially purified hyaluronic acid

The partially purified sample of hyaluronic acid was kindly supplied by Dr K. Rienits. It had been prepared from acetone-dried umbilical cords, treated with acetic acid and then extracted with 0·9% NaCl solution. Ethanol (2 vol.) was added and the precipitate was collected and re-extracted with 0·2 M-sodium acetate buffer, pH 7·0. This extract was then shaken with two separate lots of Sevag's reagent (chloroform–amyl alcohol, 2:1) and the aqueous phase precipitated with ethanol (2·5 vol.). This fraction is referred to as partially purified hyaluronic acid.

Further fractionation was carried out as follows: 2·25 g. was dissolved in 0·2 M-Na_2SO_4 (400 ml.) and 0·75 g. of cetyltrimethylammonium bromide (CTAB) was added with stirring. After standing overnight, the milky solution was centrifuged at 44 000 *g* and the supernatant was kept for the preparation of fraction K 2. The residue was dissolved in 10% (w/v) NaCl (50 ml.) and ethanol (2·5 vol.) was added with stirring. After standing for 3 hr. the precipitate (0·58 g.) was collected and dialysed against several changes of distilled water. Then 0·2 vol. of 0·6 M-sodium acetate buffer, pH 5·0, was added to the dialysed solution followed by ethanol (2·5 vol.). The mixture stood at 4° for 3 hr. and the precipitate was collected, washed with 80% (v/v) ethanol, absolute ethanol and ether to give 0·44 g. of fraction K 1. This was dissolved in 0·6 M-sodium acetate

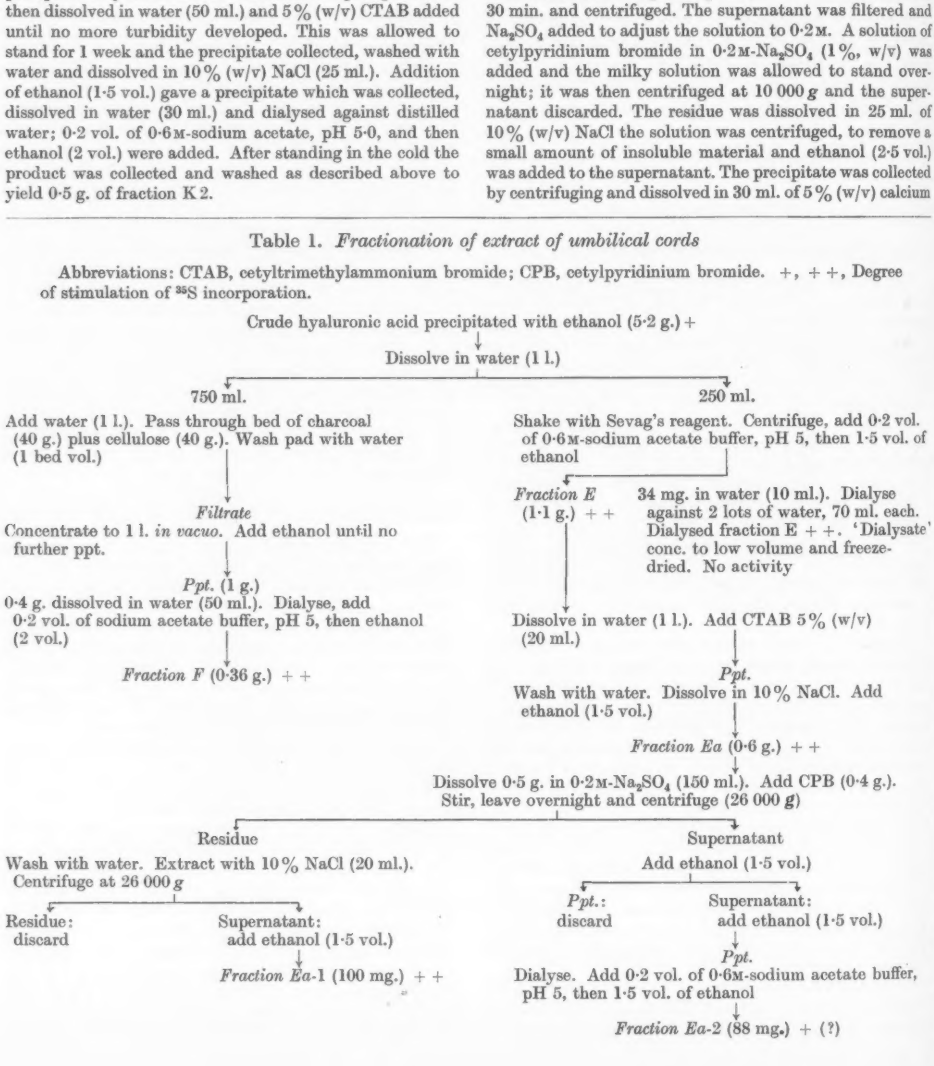
buffer, pH 5·0 (100 ml.), and ethanol (100 ml.) was added dropwise with stirring. After standing at 4° for 3 hr. the precipitate was collected and washed as above to yield 0·2 g. of fraction K 1-1. Dropwise addition of a further 50 ml. of ethanol to the supernatant gave a further precipitate which was collected and washed as above to give 0·21 g. of fraction K 1-2.

Ethanol (2 vol.) was added with stirring to the original supernatant from the treatment with CTAB. The precipitate of Na_2SO_4 was removed by centrifuging and more ethanol (1 vol.) was added with stirring to the supernatant. The precipitate was collected, dissolved in water and again precipitated by the addition of ethanol. The precipitate was then dissolved in water (50 ml.) and 5% (w/v) CTAB added until no more turbidity developed. This was allowed to stand for 1 week and the precipitate collected, washed with water and dissolved in 10% (w/v) NaCl (25 ml.). Addition of ethanol (1·5 vol.) gave a precipitate which was collected, dissolved in water (30 ml.) and dialysed against distilled water; 0·2 vol. of 0·6M-sodium acetate, pH 5·0, and then ethanol (2 vol.) were added. After standing in the cold the product was collected and washed as described above to yield 0·5 g. of fraction K 2.

Treatment of fraction K 1 *with proteolytic enzymes.* Fraction K 1 (256 mg.) was dissolved in 20 ml. of an aqueous 0·1% solution of 'Difco 1:10 000' pepsin, adjusted to pH 2 with HCl, a layer of toluene was added to the solution and it was incubated at 37° overnight. Adjustment was made to pH 8 with NaOH solution, 50 mg. of 'Difco 1:250' trypsin was added, and a layer of toluene, and the solution was incubated overnight at 37°. The solution was then dialysed with stirring for 5 hr. against two changes of 2 l. of distilled water and shaken four times with Sevag's reagent, the mixture being centrifuged between treatments and the aqueous layer was siphoned off. Lloyd's reagent (1 g.) was then added to the aqueous phase, the mixture stirred for 30 min. and centrifuged. The supernatant was filtered and Na_2SO_4 added to adjust the solution to 0·2M. A solution of cetylpyridinium bromide in 0·2M-Na_2SO_4 (1%, w/v) was added and the milky solution was allowed to stand overnight; it was then centrifuged at 10 000 *g* and the supernatant discarded. The residue was dissolved in 25 ml. of 10% (w/v) NaCl the solution was centrifuged, to remove a small amount of insoluble material and ethanol (2·5 vol.) was added to the supernatant. The precipitate was collected by centrifuging and dissolved in 30 ml. of 5% (w/v) calcium

Table 1. *Fractionation of extract of umbilical cords*

Abbreviations: CTAB, cetyltrimethylammonium bromide; CPB, cetylpyridinium bromide. +, + +, Degree of stimulation of ^{35}S incorporation.


Crude hyaluronic acid precipitated with ethanol (5·2 g.) +
Dissolve in water (1 l.)
750 ml.
250 ml.
Add water (1 l.). Pass through bed of charcoal (40 g.) plus cellulose (40 g.). Wash pad with water (1 bed vol.)
Filtrate
Concentrate to 1 l. in vacuo. Add ethanol until no further ppt.
Ppt. (1 g.)
0·4 g. dissolved in water (50 ml.). Dialyse, add 0·2 vol. of sodium acetate buffer, pH 5, then ethanol (2 vol.)
Fraction F (0·36 g.) + +
Shake with Sevag's reagent. Centrifuge, add 0·2 vol. of 0·6M-sodium acetate buffer, pH 5, then 1·5 vol. of ethanol
Fraction E (1·1 g.) + +
34 mg. in water (10 ml.). Dialyse against 2 lots of water, 70 ml. each. Dialysed fraction E + +. 'Dialysate' conc. to low volume and freeze-dried. No activity
Dissolve in water (1 l.). Add CTAB 5% (w/v) (20 ml.)
Ppt.
Wash with water. Dissolve in 10% NaCl. Add ethanol (1·5 vol.)
Fraction Ea (0·6 g.) + +
Dissolve 0·5 g. in 0·2M-Na2SO4 (150 ml.). Add CPB (0·4 g.). Stir, leave overnight and centrifuge (26 000 g)
Residue
Supernatant
Wash with water. Extract with 10% NaCl (20 ml.). Centrifuge at 26 000 g
Add ethanol (1·5 vol.)
Residue: discard
Supernatant: add ethanol (1·5 vol.)
Fraction Ea-1 (100 mg.) + +
Ppt.: discard
Supernatant: add ethanol (1·5 vol.)
Ppt.
Dialyse. Add 0·2 vol. of 0·6M-sodium acetate buffer, pH 5, then 1·5 vol. of ethanol
Fraction Ea-2 (88 mg.) + (?)

acetate containing 0·5 N-acetic acid. Ethanol (14 ml.) was added dropwise with stirring. After standing for 2 hr. at 4° the precipitate was collected. The supernatant was adjusted to 50 % (v/v) ethanol by the further dropwise addition of ethanol. After a further 2 hr. at 4°, the precipitate was collected by centrifuging, washed with 80 % (v/v) ethanol, absolute ethanol and ether. The yield was 180 mg. of fraction K 1-50. Fraction K 1-50 was identified as CSA-C by the following data: optical rotation $(\alpha)_D - 16°$; molar ratios taking hexosamine as unity, hexosamine:uronic acid: nitrogen:sulphur:acetyl, 1·0:1·1:0·97:0·81:1·2. The result of infrared examination is given in Fig. 5 (Orr, 1954). This fraction was dialysed against distilled water and reprecipitated from calcium acetate buffer with ethanol as previously described before testing for stimulating activity.

Additional quantities of CSA-C were isolated from the residues of the cords remaining after extraction with 0·9 % NaCl. Digestion with proteolytic enzymes and treatment with Sevag's reagent and Lloyd's reagent was carried out as described above. Analyses of this material and other mucosubstances, tested for their effects on [^{35}S]sulphate incorporation into CSA, are reported in Tables 2 and 3. Results obtained with CSA-C refer to this material except where specific mention is made to the use of fraction K 1-50.

Preparation and sources of other materials

Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. This was prepared by electrophoresis of the nucleotide fraction obtained by incubating -[^{35}S]sulphate with the above system (Adams, 1959*a*).

Chondroitin sulphate A. Crude CSA was isolated from bovine trachea by the method of Bera, Foster & Stacey (1955). Further purification was carried out as follows: 18 g. of sodium CSA was dissolved in water (1 l.) and 5 % (w/v) of CTAB added with stirring until no further precipitate formed. The white precipitate was collected by centrifuging and washed with two lots of water. It was then dissolved in the minimum volume of 10 % (w/v) $CaCl_2$, centrifuged to remove insoluble matter and ethanol (1·5 vol.) added to the supernatant with stirring. The precipitate which formed was collected, dissolved in 350 ml. of 5 % (w/v) calcium acetate containing 0·5 N-acetic acid and shaken with Sevag's reagent. After centrifuging, the aqueous layer was separated and ethanol added dropwise with stirring to a concentration of 30 % (v/v). More ethanol was added and two additional fractions were collected at concentrations of 40 % (v/v) and 50 % (v/v) of ethanol. The fraction precipitating at 30 % was dissolved in water (75 ml.) and precipitated with ethanol (2 vol.). It was then redissolved in water, dialysed against several changes of distilled water and precipitated with ethanol (1·5 vol.) after previous addition of calcium acetate and acetic acid as before. The precipitate was collected, washed with ethanol 80 % (v/v), absolute ethanol and ether. The yield was 1·5 g. of CSA-A. The fractions precipitating at 40 % and 50 % ethanol weighed 7·5 and 3·0 g. respectively.

With CSA-A and -C, identification of the hexosamine moiety as galactosamine was confirmed by ninhydrin degradation to lyxose (Stoffyn & Jeanloz, 1954). Final proof of the identity of CSA-A and -C was provided by infrared spectra (Fig. 5). Peaks at 928 cm.$^{-1}$ and 852 cm.$^{-1}$ characteristic of CSA-A, and at 1000 cm.$^{-1}$ and 775 cm.$^{-1}$ characteristic of CSA-C, were evident (Orr, 1954; Mathews, 1958).

Chondroitin sulphate B. This was a gift kindly supplied by Dr Albert Dorfman.

Table 2. *Analyses of fractions obtained from umbilical-cord extract and from partially purified hyaluronic acid*

Fraction	Percentage					Non-hexosamine N (by difference) (%)	Non-hexosamine N as protein (%)
	C	H	N	S	Hexosamine		
F	35·4	6·2	4·5	0·91	25·0	2·6	16
Ea-1	30·2	5·5	4·2	2·96	19·1	2·6	16
Ea-2	35·5	6·2	4·5	0·3	28·7	2·2	14
K 1-1	30·5	5·5	3·1	3·75	20·4	1·5	10
K 1-2	29·3	5·1	3·1	3·82	26·4	1·0	6
K 2	33·8	5·8	2·9	0·5	26·1	0·9	6

Table 3. *Analyses of materials tested for their effects on biosynthesis of chondroitin sulphate*

Substance	$[\alpha]_D$	Percentage					Molar ratio (hexosamine = 1·0)				Protein (non-hexosamine N × 6·25) (%)
		Hexosamine	Uronic acid	N	S	N/S	Hexosamine	Uronic acid	N	S	
CSA-A sodium salt	−34°	30·0	35·0	2·4	5·1	1·09	1·0	1·07	1·04	0·95	0·5
CSA-C sodium salt	−16°	29·0	28·5	2·4	4·7	0·88	1·0	0·91	1·04	0·91	0·6
Bovine chondromucoprotein	—	23·0	26·4	5·5	2·9	4·4	1·0	1·06	3·08	0·70	23
Chick-cartilage mucoprotein (20 days)	—	21·8	28·1	6·5	2·5	5·9	1·0	1·19	3·80	0·64	30
Hyaluronic acid from cord fractionation	—	34·8	49·5	4·2	0·63	—	1·0	1·30	1·56	0·10	9
Hyaluronic acid after alkali-treatment	—	—	—	2·78	0·17	—	—	—	—	—	—

Chondromucoprotein. Isolation of the mucoprotein from bovine nasal septum by aqueous extraction with high-speed homogenization, was carried out as described by Malawista & Schubert (1958). The pure white product had a similar analysis to that isolated by these workers (see Table 3) and treatment with dilute alkali as described furnished microcrystalline CSA.

Chick-embryo-cartilage mucoprotein. Extraction of the mucoprotein was carried out in a similar manner to that described above. Condyles from the tibias and femora of twelve 15-day-old chick embryos (wet wt. 1·8 g.) were extracted with 54 ml. of cold water in an homogenizer running at 40 000 rev./min. Mucoprotein (43 mg.) was obtained and was dissolved in water, dialysed and precipitated with 3 vol. of ethanol containing 2% (w/v) of potassium acetate. The precipitate was collected, washed with ethanol [80% (v/v) then absolute] and ether (fraction A). Further purification was carried out as follows: 34 mg. was stirred with water (10 ml.), and this was centrifuged at 4000 rev./min. and the supernatant drawn off from the residual gel. The gel was then re-extracted with water (3 ml.) and again centrifuged. To the combined supernatant, 0·5 ml. of 10% (w/v) NaCl was added followed by 1 ml. of 5% (w/v) CTAB. The solution was stirred and the precipitate collected by centrifuging, washed by suspending in two separate lots of water and then dissolved in 5 ml. of 10% (w/v) NaCl. The solution was centrifuged at 17 000 rev./min. (Spinco) and ethanol (1·5 vol.) was added, with stirring, to the clear supernatant. After cooling at 4° for 3 hr. the precipitate was collected by centrifuging and washed with ethanol [80% (v/v), then absolute] and ether to give 10·6 mg. (fraction B). Extraction of condyles of 20-day-old chick embryos was carried out in a similar manner.

Chondroitin. The material used was that described previously (Adams, 1959*a*), prepared by desulphation of CSA by the method of Kantor & Schubert (1957).

Hyaluronic acid. A sample of hyaluronic acid isolated from cords by fractionation with cetylpyridinium bromide had contents of 4·2% of N and 0·63% of S (Table 3). It was treated as follows: 0·43 g. was dissolved in 60 ml. of water and adjusted to pH 2 with HCl. Ammonium sulphate (40 g.) was added, with stirring, to the solution maintained at 5° (after Jeanloz & Forchielli, 1950). After standing some hours in the cold the fine precipitate was collected by centrifuging. This precipitate was dissolved in water (70 ml.) and the solution adjusted to 0·2N-NaOH by addition of 10N-NaOH, then placed in a water bath at 50° for 1 hr., cooled, dialysed against running tap water overnight and then against frequent changes of distilled water. Then 0·2 vol. of 0·6M-sodium acetate buffer, pH 5, was added and the solution shaken with Sevag's reagent three times, centrifuging between treatments. To the aqueous phase 5% (w/v) CTAB was added until precipitation was complete. The precipitate was collected by centrifuging, washed by suspension in two lots of water and dissolved in 10% (w/v) NaCl. Ethanol (1·5 vol.) was added with stirring and the precipitate collected after cooling. It was then dialysed, sodium acetate buffer added and precipitated with ethanol (2 vol.). The precipitate was collected and washed with ethanol and ether. The yield was 211 mg.

Treatment with hyaluronidase. Essentially the method was that of Houck & Pearce (1957), who described the degradation of hyaluronic acid and CSA to a mixture of long-chain oligosaccharides.

CSA-C (sodium salt; 110 mg.) was dissolved in 25 ml. of 0·1M-sodium acetate buffer, pH 4·8, containing 0·15M-NaCl. This was divided into three equal volumes and 0·1 ml. of the buffer solution containing 50 i.u. of testicular hyaluronidase (Rondase, Evans Medical Supplies) was added to each tube. Incubation was at 37° and the reaction was stopped after 5, 10 and 15 min. by heating at 100° for 3 min. The solutions were pooled and ethanol (25 ml.) was added slowly with stirring. After cooling, the precipitate was collected, redissolved in buffer and again precipitated with ethanol (1 vol.). It was collected and washed in the usual manner.

A similar procedure was applied to 60 mg. quantities of the sodium salts of chondroitin and hyaluronic acid. After dissolving in 12 ml. of buffer and dividing into three parts, 50 i.u. of testicular hyaluronidase were added to each tube.

Reduction of hyaluronidase-treated CSA-C with sodium borohydride was carried out after Weissmann (1955). Hyaluronidase-treated CSA-C (sodium salt, 25 mg.) was stirred with 0·2 ml. of 0·1M-sodium borohydride in 0·05M-Na_2CO_3. On stirring, the polysaccharide dissolved and H_2 was liberated. After 10 min. the solution was diluted with water (2 ml.) and acidified to pH 5 with acetic acid. Ethanol was added until a precipitate formed. This was collected by centrifuging, washed with ethanol (80%, v/v), redissolved in 3 ml. of 0·2M-sodium acetate buffer, pH 7, and precipitated by addition of ethanol (2 vol.). The precipitate was collected and washed in the usual manner. The yield was 22 mg.

Fraction K1 was treated with hyaluronidase as follows: a sample (0·2 ml.) of a solution of K1 containing 7 mg./ml. was placed in a 10 ml. centrifuge tube, 0·2 ml. of 0·2M-sodium acetate buffer, pH 4·8 was added followed by 0·2 ml. (1000 i.u.) of a solution of testicular hyaluronidase. A control containing 0·2 ml. of water in place of the enzyme was also set up. The tubes were stoppered, incubated for 2 days at 37° and then heated for 1 min. at 100°. After centrifuging at 4000 rev./min. the supernatants were separately dialysed against several changes of distilled water. The dialysed solutions were then transferred to narrow tubes and concentrated to dryness in an air stream at 40°. The residues were taken up in 0·1 ml. of water.

Analytical methods

Carbon, hydrogen, nitrogen and sulphur determinations. These were carried out by Dr Zimmerman, C.S.I.R.O., Microanalytical Department, Melbourne.

Hexosamine. This was determined on a sample of polysaccharide, hydrolysed for 5 hr. in 5N-HCl, by the Blix (1948) variation of the Elson–Morgan reaction.

Uronic acid. This was determined by the Dische (1947) method. All samples were dried *in vacuo* over P_2O_5 for 3 hr. before analysis.

Paper electrophoresis of cord fractions. Fractions (5 µl.) of 3% (w/v) solution in water were applied to dry strips of paper (Schleicher & Schüll paper 2043 B, Dassel, Germany) as small spots, allowed to dry and each end of the paper was dipped in 25 mM-citrate buffer, pH 5·5 (1·68 g. of citric acid and 5·0 g. of trisodium citrate dihydrate/l. (Gomori, 1955) until the paper was wetted to within 0·5 cm. on each side of the spot. The paper was then placed on blotting paper to absorb excess of buffer and then subjected to electrophoresis at 2·7 v/cm. for 16 hr. Staining with tolui-

dine blue was carried out by the method of Leitner & Kerby (1954), and treatment with chlorine and starch–potassium iodide by the method of Rydon & Smith (1952).

For testing stimulating activity after electrophoresis, 0·3 mg. of fraction K 1-2 was applied as a spot to a 1-in.-wide paper strip. After electrophoresis, the dried paper was cut into 4 cm. sections and each section separately eluted with water overnight by clamping the paper between glass plates and allowing elution to take place by capillarity: the end of the paper was immersed in a dish of water and the eluate allowed to drip into a small beaker. Elution was carried out in a closed container. The eluates were each separately dialysed against several changes of distilled water and then concentrated to dryness in an air stream at 40°. The residues were dissolved in water (0·1 ml.) for testing purposes.

Electrophoresis and scanning of radioactive chondroitin sulphate. After incubation with enzyme in which CSA-A and CSA-C were added to the incubation medium, which contained 10 μc of [^{35}S]sulphate, 1 ml. of 0·2 M-Na_2SO_4 and 0·1 ml. of 1% (w/v) CSA (Sigma Chemical Co.) were added to each tube. The solution was then heated for 1 min. in a boiling-water bath and 1 ml. of NaOH immediately added. After standing for 5 min. The solution was adjusted to pH 8 with N-HCl. A portion (8 ml.) of 0·2 M-Na_2SO_4 and 0·1 ml. of 5% (w/v) CTAB were then added and the solution was thoroughly mixed. The precipitate was allowed to stand overnight and then centrifuged and washed by suspension in two separate lots of 0·2 M-Na_2SO_4. The precipitate was dissolved in 10% NaCl, precipitated with ethanol and washed and dried as described above. The precipitate of free polysaccharide was dissolved by addition of 0·1 ml. of water. A sample (0·01 ml.) of solution was then applied as a small spot to strips of dry filter paper (Schleicher & Schüll paper 2043 B) and subjected to electrophoresis in 25 mM-citrate buffer, pH 5·5, at 2·7 v/cm. for 16 hr. The papers were dried and radioactive zones measured on an automatic scanner. Metachromatic areas were revealed by staining with toluidine blue (Leitner & Kerby, 1954).

Counting of radioactive samples. After incubation with adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate or $Na_2{}^{35}SO_4$, tubes were heated for 1 min. in a boiling-water bath, diluted to 10 ml. with 0·2 M-Na_2SO_4 and 1 ml. of 1% (w/v) CSA (Sigma Chemical Co.) added, then 1 ml. of 5% (w/v) CTAB. After standing overnight the precipitate was collected by centrifuging and washed by mixing with a glass rod whilst adding 5 ml. of 0·1 M-Na_2SO_4. Washings were repeated five times with 0·1 M-Na_2SO_4 and once with water. The precipitates were then collected by filtration on Whatman no. 1 paper disks. After drying in a desiccator the disks were glued to planchets and the samples counted at infinite thickness with an end-window counter having a counting efficiency of 8%. The times required to record 10^4 counts were noted. Counts at infinite thickness were converted into counts/min./mg. by using a factor determined from the following: a preparation of CSA of known radioactivity (determined as counts/min./mg. at infinite thinness) was mixed with known amounts of unlabelled material converted into the CTAB salts and the radioactivity of the mixtures was corrected to infinite thickness by using a self-absorption curve.

Reprecipitation of [^{35}S]CSA CTAB salts was carried out by dissolving the latter in 10% (w/v) NaCl and precipitating the free polysaccharide with 1·5 vol. of ethanol. After some hours at 4° the material was centrifuged, the solid was redissolved in 0·2 M-Na_2SO_4 and precipitated again by the addition of CTAB.

Infrared spectra. These were determined in Nujol (liquid paraffin) mull on a Perkin–Elmer instrument model 21.

RESULTS

Fractionation of the crude cord extract as outlined in Table 1 was designed to attempt a separation of minor constituents and also to provide a pure sample of hyaluronic acid. Treatment with cetylpyridinium bromide in 0·2 M-sodium sulphate was based on the separation of CSA from hyaluronic acid as described by Scott (1955).

Analyses of the fractions obtained is given in Table 2, together with analyses of fractions obtained by similar treatment of a partially purified sample of hyaluronic acid. These further fractions are listed as K 1-1, K 1-2 and K 2 (see Methods section).

When tested for ability to stimulate CSA synthesis in the chick-cartilage-enzyme system, it was found that the fraction soluble in 0·2 M-sodium sulphate (fraction Ea-2), containing only 0·3% of sulphur, had very little effect, whereas strong stimulation was associated with the sulphur-containing fractions. Active fractions are marked with a + or ++ in Table 1. That nucleotides were not responsible for the stimulation was suggested by the finding (Table 1) that the filtrate from the charcoal pad had no absorption at 260 mμ, and from this filtrate fraction F was prepared which was strongly stimulating. No sharp separation of sulphur-containing compounds from the hyaluronic acid was achieved by charcoal treatment; fraction F still contained almost 1% of sulphur. Elution of the charcoal with 50% (v/v) ethanol containing 4% of ammonia gave a product which stimulated and which did not contain free reducing sugars or free hexosamine derivatives as shown by paper chromatography (butanol–acetic acid–water, 50:12:25), but gave strong metachromatic zone at the origin. On paper electrophoresis negative tests were obtained for reducing sugars and hexosamine and u.v.-absorbing zones were absent. A metachromatic band was obtained with a mobility about 0·6 times that of pure CSA-A.

Jeanloz & Forchielli (1950) have described a method for preparing hyaluronic acid comparatively free of sulphur-containing polysaccharides by fractionation with ammonium sulphate. This method was tried on a separate cord extract but was inferior to the fractionation with cetylpyridinium bromide.

When subjected to electrophoresis on paper in citrate buffer, pH 5·5, the sulphur-rich fractions gave metachromatic zones with mobilities 0·5–0·6 times that of pure CSA-A. No zone corresponding to

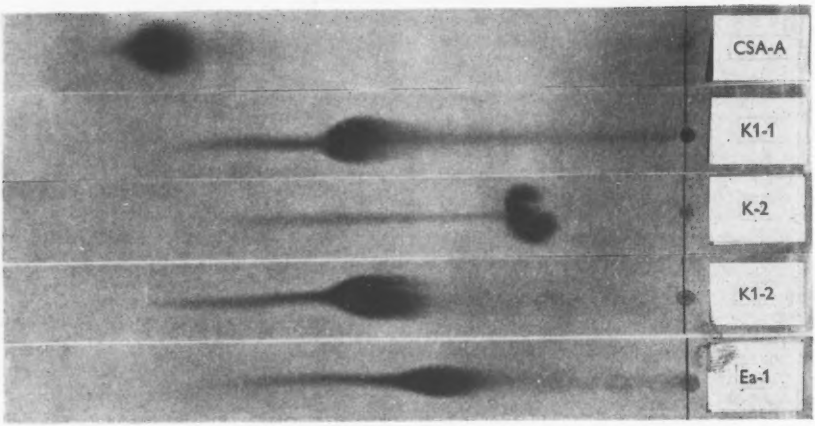


Fig. 1. Electrophoresis of fractions on paper in 25 mM-citrate buffer, pH 5·5, at 2·7 v/cm. for 16 hr. stained with toluidine blue. Pure CSA-A was run at the same time for comparison.

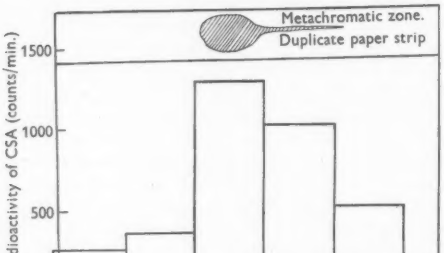


Fig. 2. Effect of eluates from sections of electrophoresis paper of fraction K 1-2 on the radioactivity of CSA formed on incubation with chick-embryo-cartilage enzyme. A duplicate paper strip stained with toluidine blue shows the position of the metachromatic zone.

pure CSA was obtained (Fig. 1). The low sulphate containing fraction, K-2, obtained by fractionation of partially purified hyaluronic acid, had a lower mobility and gave an orthochromatic spot. When the electrophoresis was carried out in barbiturate buffer, pH 8·6 (1·84 g. of barbital and 10·30 g. of sodium barbital/l.), K 1-1, K 1-2 and Ea-1 all moved more slowly than pure CSA-A but their mobilities relative to CSA-A were increased. All fractions, including pure CSA-A, left faint residual metachromatic zones at the origin which may be caused by placing the solution on dry paper. It was necessary to do this in order to obtain discrete

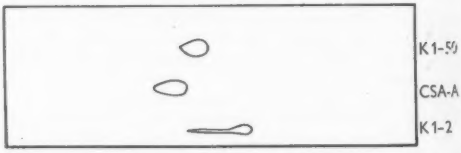


Fig. 3. Electrophoresis of fraction K 1-2, fraction K 1-50 (obtained from fraction K 1 by treatment with proteolytic enzymes) and CSA-A in 25 mM-citrate, pH 5·5. Stained with toluidine blue.

zones; these could not be obtained by spotting on to wet paper.

Fraction K 1-1 was subjected to electrophoresis on paper in 0·05 M-sodium acetate buffer, pH 5·6 (0·28 ml. of acetic acid and 3·7 g. of sodium acetate/l.), and excess of buffer removed by washing in ethanol.

On treatment with chlorine followed by starch-potassium iodide, a zone appeared which was superimposable with that obtained with toluidine blue. Both reagents also showed small zones at the origin. The chlorine reagent gave positive tests with pure CSA and hyaluronic acid on the electrophoresis paper. Free protein appeared to be absent, however, since no additional zones were revealed with the chlorine reagent.

Proof that stimulating power was due to the metachromatic zone was obtained by cutting the electrophoresis paper of fraction K 1-2 into sections, eluting each section and testing the dialysed eluate for stimulating power. Results are shown in Fig. 2.

Table 4. *Effect of addition of cord fractions on specific activity of chondroitin sulphate formed on incubation with chick-cartilage enzyme and isolated by addition of* 10 *mg. of carrier chondroitin sulphate* (Sigma)

The system comprised: ATP (5 μmoles), $MgCl_2$ (6 μmoles), cysteine hydrochloride (3 μmoles), tris buffer, pH 8·2 (60 μmoles), and enzyme (0·3 ml.); 0·7 mg. of cord fraction was tested in each case. The total volume was 0·8 ml. Incubation time was 1·5 hr. at 37°.

Expt. no.	Tracer	Fraction added	Radioactivity of chondroitin sulphate (CTAB salt): Specific activity (counts/min./mg. of CSA)	Radioactivity of chondroitin sulphate (CTAB salt): Relative to control as 1·0
1	25 μc of $Na_2{}^{35}SO_4$	Control	3 330	1·0
		E	5 740	1·7
		Ea	6 250	1·9
2	25 μc of $Na_2{}^{35}SO_4$	Control	4 040	1·0
		Charcoal eluate	8 850	2·2
		F	10 700	2·6
		Ea	10 700	2·6
		Ea-1	12 350	3·1
		Ea-2	4 900	1·2
		K1	14 700	3·6
3	16 μc of $Na_2{}^{35}SO_4$	Control	4 270	1·0
		K1	12 000	2·8
		K1-1	12 700	2·9
		K1-2	9 600	2·2
4	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate; 20 000 counts/min.	Control	71	1·0
		K1-2	171	2·4

Treatment of fractions K1 and Ea-1 containing high percentages of sulphur, with proteolytic enzymes yielded a mucopolysaccharide which had a similar, but not identical, electrophoretic mobility on paper to CSA-A (Fig. 3). Analysis, optical rotation and infrared spectral data (Fig. 5) proved it to be CSA-C. The latter was first shown to be a constituent of human umbilical cords by Meyer & Palmer (1936).

Table 4 (Expts. 1 and 2) illustrates the stimulating activity on CSA synthesis of the fractions isolated from the cords. In Expts. 3 and 4 activity of fractions, obtained by fractionation of the partially purified hyaluronic acid, are recorded.

Fig. 4 compares the stimulating activity of fractions Ea-1, K1-2 and K1-50 (CSA-C) over a range of concentrations. The response obtained with any particular fraction was found to vary with the particular enzyme preparation. Some variation in degree of activity was also found from experiment to experiment, making it necessary to compare two or more fractions in the one experiment. The variation in response with different enzyme preparations is thought to be related to the ratio of the concentration of preformed precursors and the concentration of CSA extracted from the embryonic cartilage. A second preparation of CSA-C was isolated by enzymic digestion of the cord residues remaining after the initial extraction with 0·9% sodium chloride solution. This sample gave the following analysis; hexosamine:uronic acid:nitrogen:sulphur, 1·0:0·91:1·04:0·91, and stimulated to the same degree as fraction K1-50.

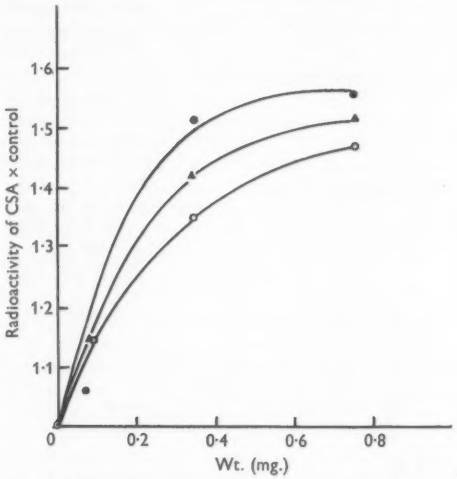


Fig. 4. Comparison of fractions Ea-1 (▲), K1-2 (●) and K1-50 (CSA-C) (○) for ability to stimulate the synthesis of CSA when incubated with the chick-embryo enzyme.

Activity of isolated chondroitin sulphates A and C

Comparison of the two isomeric CSA compounds for effect on CSA biosynthesis in the chick-enzyme system revealed that CSA-A possessed very little activity in comparison with CSA-C. This was evident when either [^{35}S]sulphate or adenosine

Table 5. *Effect of addition of mucopolysaccharides on the specific activity of chondroitin sulphate in the system as shown in Table 4*

Amount of polysaccharide added was 0·7 mg., except in Expt. 6, where it was 1·4 mg. The total volume was 0·8 ml.

Expt. no.	Tracer	Substance added	Radioactivity of CSA: Initial precipitate — Specific activity (counts/min./mg. of CSA)	Initial precipitate — Relative to control	Reprecipitation — Specific activity (counts/min./mg. of CSA)	Reprecipitation — Relative to control
1	$Na_2{}^{35}SO_4$, 10 μc	—	1690	1·0	—	—
		CSA-A, Na salt	2180	1·3	—	—
		CSA-C, Na salt	3000	1·8	—	—
	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate; 22 000 counts/min.	—	37·2	1·0	—	—
		CSA-A, Na salt	41·5	1·1	—	—
		CSA-C, Na salt	66·0	1·9	—	—
2	$Na_2{}^{35}SO_4$, 10 μc	—	5300	1·0	2740	1·0
		CSA-A, Na salt	6000	1·1	3640	1·3
		CSA-C, Na salt	7350	1·5	5100	1·9
3	$Na_2{}^{35}SO_4$, 10 μc	—	1360	1·0	573	1·0
		CSA-A, Na salt	1480	1·1	890	1·5
		CSA-A + C, Na salt (50% ethanol fraction from trachea CSA)	2200	1·6	1310	2·3
4	$Na_2{}^{35}SO_4$, 10 μc	—	1540	1·0	665	1·0
		CSA-C, Na salt	2600	1·7	1920	2·9
		Bovine mucoprotein	1260	0·8	725	1·0
		Chick mucoprotein (20 days) fraction A	1250	0·8	660	1·0
5	$Na_2{}^{35}SO_4$, 10 μc	—	2960	1·0	—	—
		Chick mucoprotein (20 days) fraction B	3120	1·0	—	—
		Chick mucoprotein (15 days) fraction B	3100	1·0	—	—
6	$Na_2{}^{35}SO_4$, 10 μc	—	2500	1·0	—	—
		Fraction K1 hyaluronidase treated and dialysed	3480	1·4	—	—
		Fraction K1 control	4900	2·0	—	—

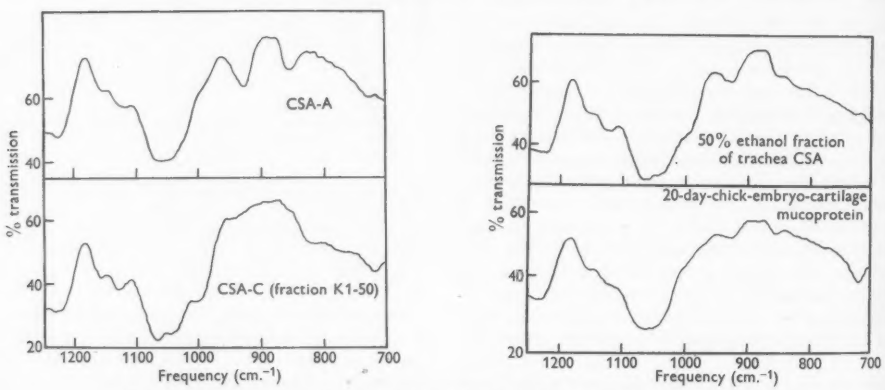


Fig. 5. Infrared spectra of chondroitin sulphates and chick-cartilage mucoprotein.

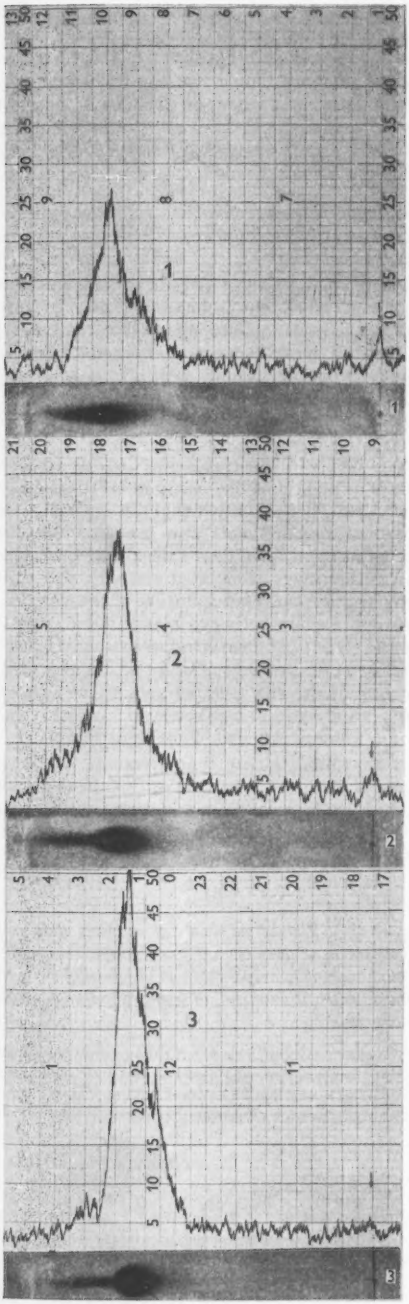


Fig. 6. For legend see next column.

3′-phosphate 5′[^{35}S]-sulphatophosphate was used as tracer. It was thought that this may have been due to the fact that CSA-A had been isolated from trachea by hot alkali and would have suffered greater degradation than CSA-C which had been extracted with cold 0·9 % sodium chloride solution and had no alkali-treatment at any stage. However, when CSA-C was treated with alkali under the conditions used for isolating CSA-A, no loss of stimulating ability was evident. The reality of the responses obtained with the two CSA compounds was further illustrated by the finding that the more soluble ethanol fraction (50 %) obtained from the separation of the trachea CSA mixture, was strongly stimulating. This fraction contains the CSA-C present in the mixture (Meyer *et al.* 1956), and was further shown to consist of CSA-A and C, the former predominating, by its infrared spectrum (Fig. 5). Chondromucoprotein obtained from bovine nasal septum was found to have a slight inhibitory action. These results are given in Table 5.

In order to prove that incubation in the presence of added CSA-C affected sulphated mucopolysaccharide synthesis, incubations were carried out in the normal way and polysaccharide was collected with the aid of a small amount of carrier CSA. Treatment with warm alkali split the protein–polysaccharide complexes, and the isolated polysaccharide was subjected to electrophoresis on paper. Fig. 6 shows the radioactive zones obtained. Radioactivity is associated with the CSA metachromatic zones only and substantial stimulation is achieved with added CSA-A (1·7 times control) and CSA-C (2·1 times control). Since the samples for electrophoresis had been subjected to a reprecipitation from solution by ethanol (see section 'Reprecipitation of ^{35}S-labelled chondroitin sulphate' below), the degree of stimulation is greater than is observed at first precipitation.

Examination of chick-embryo-cartilage mucoprotein

Comparison of the infrared spectra of chick-cartilage mucoprotein with the spectra of pure CSA-A and CSA-C indicated that it was composed essentially of CSA-A. Weak bands at 1000 cm.$^{-1}$ and 820 cm.$^{-1}$ were suggestive of CSA-C (Fig. 5). Only very slight differences were observed in the spectra of the mucoprotein from 15- and 20-day-old embryos. When ethanol precipitated mucoprotein from 15-day-old embryos (fraction A) was tested for its effect on CSA biosynthesis, some inhibition resulted. However, when this material was further purified by formation of, and regeneration from, its

Fig. 6. Scan of paper electrophoresis pattern of CSA isolated by addition of 1 mg. of carrier after incubation in the medium described in Table 4. The paper is stained with toluidine blue. 1, Control; 2, incubated in the presence of 0·7 mg. of CSA-A; 3, incubated in the presence of 0·7 mg. of CSA-C.

CTAB salt, it was found to have no effect on CSA biosynthesis. This was also found with mucoprotein derived from 20-day-old embryos (Table 5). Neither of the samples of mucoprotein moved from the origin during paper electrophoresis in 25 mM-citrate buffer, pH 5·5. Analysis (Table 3) of the mucoprotein of 20-day-old embryos showed that it contained 30 % of protein.

Reprecipitation of ^{35}S-labelled chondroitin sulphate

Radioactive CSA was isolated from the incubation mixture with the aid of carrier CSA and precipitation with CTAB. When such samples were redissolved in 10 % (w/v) sodium chloride and precipitated as free polysaccharide by the addition of ethanol and this in turn was precipitated as the CTAB salt and again counted, a loss in radioactivity resulted. CSA of constant specific activity was obtained after a further reprecipitation. Fig. 7 shows the effect of repeated precipitation on the specific activity of [^{35}S]CSA formed by incubation in the presence of CSA-A and CSA-C. At constant specific activity, CSA-C gave a stimulation of 3·95 times that of the control, whereas CSA-A stimulated to the extent of 1·76 times the control. The greater drop in specific activity after one reprecipitation when compared with that shown in Table 5 is due to the use of more than twice the usual amount of carrier in the isolation of the [^{35}S]CSA in the experiment illustrated in Fig. 7. This alteration was made in order to provide enough material for the repeated precipitations.

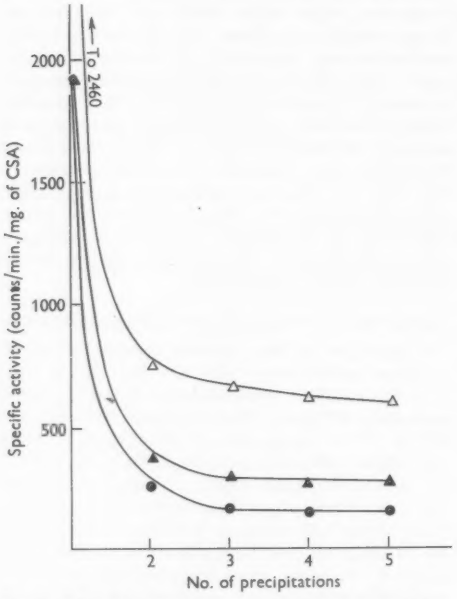


Fig. 7. Effect of reprecipitation on specific activity of CSA isolated from system as given in Table 4 but on three times the scale and with addition of 70 mg. of carrier CSA (Sigma Chemical Co.). ●, Control; ▲, incubation in the presence of 1·4 mg. of CSA-A; △, incubation in the presence of 1·4 mg. of CSA-C.

Activity of isomeric chondroitin sulphates in a chick-condyle extract treated so as to remove acid mucopolysaccharides

Results have indicated that if removal of the sulphated mucopolysaccharides from the chick-cartilage extract could be achieved without damage to the CSA-synthesizing system, then CSA-A, and CSA-C if added, would possibly possess equal stimulating ability. An attempt was made to remove acid mucopolysaccharides from the extract by treatment with protamine (Chargaff & Olson, 1937). Table 6 illustrates the results obtained. CSA-B, which does not occur in cartilage (Meyer *et al.* 1956) showed only a slight stimulating effect when the measurements were made on the initial precipitate of CSA CTAB salt. Both CSA-A and CSA-C, however, stimulated strongly and to the same degree. On reprecipitation of the samples to constant specific activity, it was found that the specific activity of the control was reduced by 90 %, whereas in the presence of CSA-A, CSA-B or CSA-C the specific activity was reduced by 30 % in each case.

Chain length and stimulating activity

If a priming rather than template mechanism operates in the biosynthesis of CSA in the system studied, an increase in stimulating activity would be expected if the number of end groups available for attachment of precursors was increased. CSA-C, chondroitin and hyaluronic acid are all substrates

Table 6. *Effect of addition of sodium chondroitin sulphates A, B and C on [^{35}S]sulphate incorporation into chondroitin sulphate in a chick-condyle extract treated with protamine to remove acid mucopolysaccharides*

The system is as in Table 4, but the amount of CSA isomer added was 2·8 mg. in each case and the amount of carrier-free $Na_2{}^{35}SO_4$ was 9 μc. The total volume was 1·1 ml. See text for preparation of protamine-treated condyle extract.

Substance added	Specific activity (counts/min./mg. of CSA)	
	Initial precipitate	Reprecipitated to constant activity
—	385	45
CSA-A	1165	800
CSA-B	450	310
CSA-C	1235	880

for hyaluronidase. Treatment of these mucopolysaccharides with the enzyme was carried out in such a way as to produce a preparation composed of a mixture of long-chain oligosaccharides. In order to compare the C-4 hexosamine epimers, chondroitin and hyaluronic acid, a sample of the latter, derived from umbilical-cord fractionation, was treated with warm alkali for a short time and then with Sevag's reagent in order to remove any protein. Such treatment had been used to extract the CSA from trachea; the CSA was then desulphated to chondroitin.

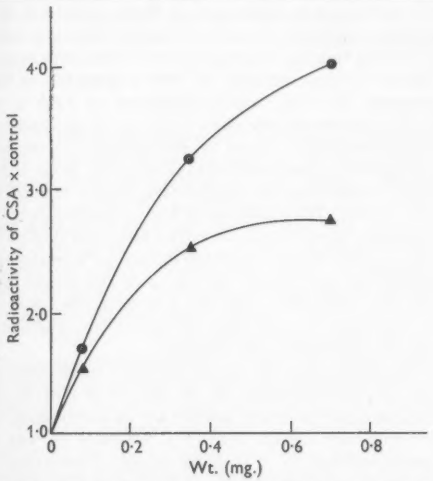


Fig. 8. Effect of depolymerization of CSA-C on ability to stimulate [^{35}S]sulphate uptake into CSA. ▲, CSA-C; ●, CSA-C after short treatment with hyaluronidase.

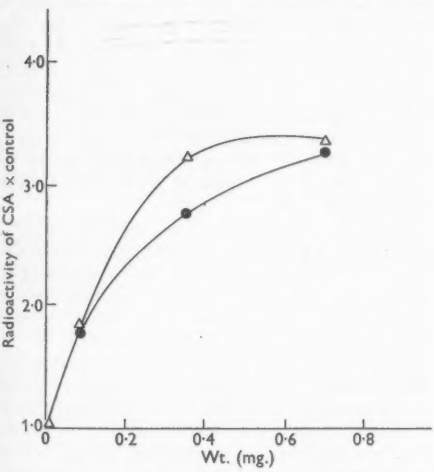


Fig. 9. Effect of reduction of terminal aldehyde groups on stimulating activity. ●, CSA-C treated with hyaluronidase; ▲, CSA-C treated with hyaluronidase and then reduced with sodium borohydride.

A considerable increase in stimulating activity resulted after hyaluronidase treatment of CSA-C (Fig. 8). Reduction of terminal aldehyde groups of this preparation with sodium borohydride (Weissmann, 1955) did not affect stimulating activity, which indicated that terminal aldehyde groups were not involved in the mechanism (Fig. 9). Chondroitin, which had a slight inhibitory effect, was found to possess stimulating activity after hyaluronidase treatment. Hyaluronic acid before enzyme treatment showed a negligible stimulating action. Some activity was produced on treatment with hyaluronidase (Fig. 10). In another experiment the CSA-C mucoprotein isolated from umbilical cords (fraction K 1) was incubated with hyaluronidase for 2 days and dialysed. The dialysed material was found to retain 40 % of its stimulating activity (Table 5).

DISCUSSION

Incorporation of [^{35}S]sulphate into CSA *in vivo* and *in vitro* has been shown to reflect synthesis of CSA (Bostrom & Aquist, 1952; Bostrom & Mansson, 1953; D'Abramo & Lipmann, 1957; Dorfman & Schiller, 1958). Effects of various suspected

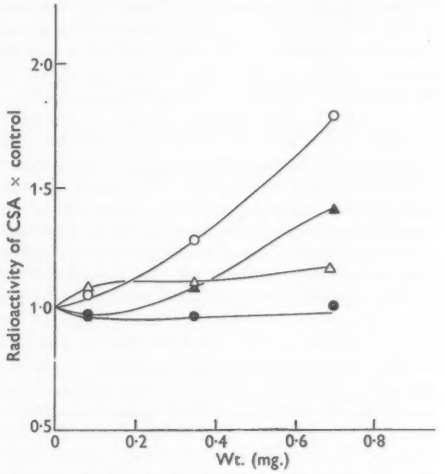


Fig. 10. Influence of chondroitin and hyaluronic acid on [^{35}S]sulphate uptake into CSA. ●, Chondroitin; ○, chondroitin after short treatment with hyaluronidase; △, hyaluronic acid; ▲, hyaluronic acid after short treatment with hyaluronidase.

intermediates, hormones etc. on CSA synthesis can then be gauged by measuring the [^{35}S]sulphate incorporation into the CSA molecule.

The comparatively mild extraction procedures used to isolate the cord fractions in the present work has resulted in products which contain protein apparently firmly bound to mucopolysaccharide. Because of the low paper-electrophoretic mobility of these fractions, it was at first thought that the metachromatic zones might represent sulphate-deficient mucopolysaccharides and this in turn would offer an explanation of their stimulating activity, i.e. they could act as sulphate acceptors. However, this was ruled out by the finding that the action of proteolytic enzymes gave only one mucopolysaccharide, CSA-C. Since stimulating activity was found to reside within the metachromatic zone, and furthermore since CSA-C was found to possess approximately the same degree of stimulating activity as the fraction from which it was derived, such activity must reside with the mucopolysaccharide itself. Thus a priming or perhaps a template mechanism appears to be operating in the biosynthesis of CSA. Since fractions Ea-1 and K1, which contain protein, stimulate as much as the free mucopolysaccharide, binding to protein is unlikely to interfere with this priming or template mechanism.

The presence of CSA-A as the major component of the embryonic-chick cartilage offers an explanation for the difference in stimulating activity between CSA-A and C. If we presume that enzymes are present which are capable of forming both types of CSA, then addition of CSA-C would be expected to show a greater effect than CSA-A, since the enzyme system as extracted would be rich in this latter type of component. An alternative explanation would be that CSA-C is the precursor or template for CSA-A formation. Bovine-cartilage mucoprotein, chick-embryo-cartilage mucoprotein, chondroitin and hyaluronic acid failed to show significant stimulating activity. Since CSA-C possessed a greater stimulating activity on decreasing the chain length by brief treatment with hyaluronidase and thereby increasing the number of end groups, a priming mechanism is indicated. Attachment of mono- or oligo-saccharide units to preformed chains, as governed by the enzyme concerned, must be specific and dependent on the position of the sulphate group in the primer molecule, otherwise no difference would have been found in priming power between CSA-A and CSA-C. Of the isomeric chondroitin sulphates, CSA-A and CSA-B have sulphate groups on position 4 of the galactosamine, whereas it is on position 6 of the galactosamine in CSA-C (Mathews, 1958; Jeanloz & Stoffyn, 1958; Hoffman, Linker & Meyer, 1958). Further, iduronic acid has been identified as the uronic acid moiety in CSA-B in contrast with glucuronic acid in both CSA-A and CSA-C (Hoffman, Linker & Meyer, 1956; Cifonelli, Ludowieg & Dorfman, 1958).

The finding that CSA-A and CSA-C possessed equal stimulating activity when tested with an enzyme extract rendered deficient in acid mucopolysaccharides confirms the above suggestions for the difference in behaviour of these isomers in the normal cartilage extract. Results obtained with CSA-B in this mucopolysaccharide-deficient extract (Table 6) can perhaps be best explained by the presence of very small quantities of CSA-A or CSA-C in this preparation. Such small amounts of CSA-A or CSA-C could be enzymically condensed with radioactive chains, thus altering their solubility relative to the radioactive chains formed in the control. This question of loss in radioactivity is discussed more fully below. The failure of CSA-B to replace the CSA-A or CSA-C isomer as a primer, as shown by the specific activities of the initial precipitates, indicates that the specificity required of the primer molecule also applies to the configuration in the uronic acid moiety.

Further support for a type-specific mechanism of priming action is lent by the failure of both chondroitin and hyaluronic acid to act as stimulators for [^{35}S]sulphate uptake into CSA, whether by way of direct sulphation or priming action. Reduction of terminal aldehyde groups of hyaluronidase-treated CSA-C had no effect on activity, indicating that the point of attachment of mono- or oligo-saccharides would be the 4 hydroxyl group of the terminal uronic acid residue. The activity possessed by chondroitin and hyaluronic acid after short treatment with hyaluronidase is most probably due to the low percentage of CSA still contained in the preparations. Evidence that the stimulation achieved by addition of CSA-A or CSA-C is not due to an enzymic exchange of [^{35}S]sulphate with the sulphate group of the polysaccharide will be presented later (J. B. Adams & K. G. Rienits, 1959, unpublished work).

Loss in radioactivity on reprecipitation of counted CSA samples is thought to be due to a difference in chain length, and hence a related difference in solubility, between radioactive CSA molecules and the CSA used as a carrier. After the first precipitation, the specific activity of both control samples and those formed by incubation in the presence of CSA-A and CSA-C became constant on repeated precipitation. It is significant that the loss in radioactivity is higher in the former case and further suggests that a priming mechanism or build-up through intermediate fragments is taking place. Attachment of these shorter radioactive chains to the primer molecule results in a molecular species similar to that used as carrier. Loss in radioactivity

on reprecipitation of hyaluronic acid, formed by extracts of Rous sarcoma incubated in the presence of ^{14}C-labelled uridine diphospho-*N*-acetylglucosamine, was observed by Glaser & Brown (1955). In this case material of constant specific activity could not be obtained. Dorfman, Markovitz & Cifonelli (1958), however, found no loss in radioactivity on repurification of hyaluronic acid formed by an extract of group A streptococci incubated in the presence of tritiated uridine diphosphoglucuronic acid.

SUMMARY

1. Crude hyaluronic acid from human umbilical cords stimulated the synthesis of chondroitin sulphate in a particle-free enzyme system derived from embryonic-chick cartilage. This stimulation was demonstrated to be due to a complex of chondroitin sulphate C and protein present in the umbilical cords. Treatment with proteolytic enzymes gave the free mucopolysaccharide, which retained the stimulating activity.

2. Chondroitin sulphate C stimulated chondroitin sulphate synthesis to a greater degree than chondroitin sulphate A. The mucoprotein isolated from the embryonic-chick cartilage was composed of 70 % of mucopolysaccharide and 30 % of protein. Infrared data showed that the former was mainly chondroitin sulphate A and probably contained small quantities of the C isomer. This mucoprotein, bovine-nasal-cartilage mucoprotein, chondroitin and hyaluronic acid, failed to stimulate chondroitin sulphate synthesis.

3. Upon treatment of the chick-cartilage extract with protamine to remove acid mucopolysaccharides, chondroitin sulphate A and C possessed equal stimulating power when tested with this extract. This indicates that relative saturation of the normal cartilage extract with chondroitin sulphate A-type components accounts for the higher degree of activity obtained with chondroitin sulphate C. Chondroitin sulphate B, which does not occur in cartilage, could not replace chondroitin sulphate A or C in ability to stimulate [^{35}S]sulphate incorporation into chondroitin sulphate in the mucopolysaccharide-deficient extract.

4. Depolymerization of chondroitin sulphate C by short treatment with testicular hyaluronidase led to a marked increase in stimulating activity. Reduction of terminal aldehyde groups of this preparation did not alter its activity.

5. A priming mechanism of synthesis of isomeric chondroitin sulphates is proposed which is type-specific.

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REFERENCES

Adams, J. B. (1959*a*). *Biochim. biophys. Acta*, **32**, 559.
Adams, J. B. (1959*b*). *Nature, Lond.*, **184**, 274.
Bera, B. C., Foster, A. & Stacey, M. (1955). *J. chem. Soc.* p. 3788.
Blix, G. (1948). *Acta chem. scand.* **2**, 467.
Bostrom, H. & Aquist, S. E. G. (1952). *Acta chem. scand.* **6**, 1557.
Bostrom, H. & Mansson, B. (1953). *Acta chem. scand.* **7**, 1014.
Chargaff, E. & Olson, K. B. (1937). *J. biol. Chem.* **122**, 153.
Cifonelli, J. A., Ludowieg, J. & Dorfman, A. (1958). *J. biol. Chem.* **233**, 541.
D'Abramo, F. & Lipmann, F. (1957). *Biochim. biophys. Acta*, **25**, 211.
Davidson, E. A. & Meyer, K. (1954). *J. biol. Chem.* **211**, 605.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dorfman, A., Markovitz, A. & Cifonelli, J. A. (1958). *Fed. Proc.* **17**, 1093.
Dorfman, A. & Schiller, S. (1958). *Recent Progr. Hormone Res.* **14**, 427.
Glaser, L. F. & Brown, D. H. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 253.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hoffman, P., Linker, A. & Meyer, K. (1956). *Science*, **124**, 1252.
Hoffman, P., Linker, A. & Meyer, K. (1958). *Biochim. biophys. Acta*, **30**, 184.
Houck, J. C. & Pearce, R. H. (1957). *Biochim. biophys. Acta*, **24**, 607.
Jeanloz, R. W. & Forchielli, E. (1950). *J. biol. Chem.* **186**, 495.
Jeanloz, R. W. & Stoffyn, P. J. (1958). *Fed. Proc.* **17**, 249.
Kantor, T. G. & Schubert, M. (1957). *J. Amer. chem. Soc.* **79**, 152.
Leitner, J. G. & Kerby, G. P. (1954). *Stain Tech.* **29**, 257.
Malawista, I. & Schubert, M. (1958). *J. biol. Chem.* **230**, 535.
Mathews, M. B. (1958). *Nature, Lond.*, **181**, 421.
Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
Meyer, K. & Palmer, J. W. (1936). *J. biol. Chem.* **114**, 689.
Orr, S. F. D. (1954). *Biochim. biophys. Acta*, **14**, 173.
Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
Scott, J. E. (1955). *Chem. & Ind.* p. 168.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **53**, 373.
Weissmann, B. (1955). *J. biol. Chem.* **216**, 783.

Biochem. J. (1960) **76**, 534

Some Observations on the Kinetics of the Reactions with Gases of Natural and Reconstituted Haemoglobins

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The first experiments in which protohaem was removed from haemoglobin and the native globin used to prepare haemoglobins containing protohaem, mesohaem and deuterohaem were reported by Hill & Holden (1926). The only reports of work on the oxygen equilibria of reconstituted protohaemoglobin which we have been able to trace appear to be those of Hill, quoted by Lemberg & Legg (1949), and of Davies, quoted by Wyman (1948). Hill found that the oxygen-dissociation curve was hyperbolic, a finding confirmed by Davies, who also found that the oxygen pressure for half-saturation was decreased to about one-quarter of that required for natural haemoglobin, and that the Bohr effect was absent.

More recently Rossi-Fanelli, Antonini & Caputo (1958) have prepared globin from human haemoglobin which could be coupled with protohaematin to give a product having the same absorption spectrum, sedimentation constant and electrophoretic behaviour as natural human methaemoglobin. After reduction by means of an enzyme system the oxygen-dissociation curve was the same as that for natural haemoglobin; the oxygen concentration for half-saturation, the degree of inflexion of the curve and the Bohr effect all agreed closely (Rossi-Fanelli & Antonini, 1959*a*).

Although the evidence presented by Rossi-Fanelli & Antonini (1959*a*) suggests strongly that haem–haem interaction had survived unchanged in the reconstituted protein, the form of the dissociation curve over the middle range of saturations is not, by itself, fully conclusive. Thus Roughton (1949) has pointed out that on the Adair (1925) hypothesis many sets of equilibrium constants describing the consecutive reactions of oxygen molecules with reduced haemoglobin can be found which yield dissociation curves scarcely differing from each other or from a given model over the range 10–90% saturation. Values of the individual equilibrium constants can, so far, only be obtained by exacting gasometric procedures which require prolonged training and experience of the operator (see, for example, Roughton, Otis & Lyster, 1955). Procedures have, however, been devised which will allow the determination of several kinetic constants corresponding to individual partial reactions of haemoglobin with gases. Such determinations offer not only information about haem–haem interaction similar to that embodied in the values of the individual equilibrium constants, but in addition give a further insight into the mechanism of the reactions.

In this paper it is shown that reconstituted human protohaemoglobin prepared by the methods of Rossi-Fanelli *et al.* (1958) gives the same values for the individual kinetic constants as the natural protein in every case which we have been able to examine. Comparative measurements have also been made on haemoglobins in which mesohaem and deuterohaem have replaced protohaem, thus extending the work already initiated by Rossi-Fanelli & Antonini (1959*b*), and Rossi-Fanelli, Antonini & Caputo (1959) in the kinetic field.

EXPERIMENTAL

Notation. According to the intermediate-compound hypothesis of Adair (1925) the reaction between haemoglobin (Hb) and oxygen (or any other ligand) takes place in four stages: the kinetic and equilibrium constants may be written as shown.

$$\begin{aligned} &Hb_4 + O_2 \rightleftharpoons Hb_4O_2 && K_1 = k_{01}/k_{10} \\ &Hb_4O_2 + O_2 \rightleftharpoons Hb_4(O_2)_2 && K_2 = k_{12}/k_{21} \\ &Hb_4(O_2)_2 + O_2 \rightleftharpoons Hb_4(O_2)_3 && K_3 = k_{23}/k_{32} \\ &Hb_4(O_2)_3 + O_2 \rightleftharpoons Hb_4(O_2)_4 && K_4 = k_{34}/k_{43} \end{aligned}$$

The reactions with carbon monoxide are dealt with in the same way except the letters L and l are used instead of K and k to distinguish them from the oxygen reactions.

Materials. Protohaemin was obtained from British Drug Houses Ltd.; deuterohaemin was prepared by the method of Fischer & Orth (1940*a*); mesohaemin was prepared by the method of Erdmann & Corwin (1947); mesoporphyrin was prepared from protohaemin by the method of Fischer & Orth (1940*b*); human globin was prepared by the method of Rossi-Fanelli *et al.* (1958); carbon monoxide was supplied by Imperial Chemical Industries Ltd.

Buffers. All reactions were carried out in 0·1 M-phosphate buffer, pH 7·1, made by mixing 5 vol. of 0·1 M-Na_2HPO_4 with 2 vol. of 0·1 M-KH_2PO_4.

Reconstituted haemoglobins. These were prepared and reduced with the enzyme system of Rossi-Fanelli & Antonini (1959*a*).

Concentrations of haem pigments. These were determined as the CO compounds with a Unicam SP. 600 spectrophoto-

Table 1. *Reactions of haemoglobin with oxygen and carbon monoxide*

All determinations were carried out at 20·5° in 0·1 M-phosphate buffer, pH 7·1, with total pigment concentrations of: 3 μM for k_{43} and l_{43}; 40 μM for k_{34} and l_{34}; 10 μM for spectrophotometric determination of M. Values of K_4 and L_4 were calculated from the kinetic constants and M from K_4 and L_4. Units: combination velocity constants, M^{-1} sec.$^{-1}$, based on haemin iron; dissociation velocity constants, sec.$^{-1}$; equilibrium constants, M^{-1}.

	Natural protohaemoglobin	Reconstituted protohaemoglobin	Mesohaemoglobin	Deuterohaemoglobin
k_{34}	$3{\cdot}2 \times 10^7$	$3{\cdot}0 \times 10^7$	$1{\cdot}4 \times 10^7$	$5{\cdot}7 \times 10^7$
k_{43}	34	34	8	60
l_{34}	$7{\cdot}0 \times 10^6$	$7{\cdot}0 \times 10^6$	$7{\cdot}0 \times 10^6$	$8{\cdot}8 \times 10^6$
l_{43}	0·031	0·031	0·022	0·038
K_4	$9{\cdot}4 \times 10^5$	$8{\cdot}8 \times 10^5$	$3{\cdot}0 \times 10^6$	$9{\cdot}5 \times 10^5$
L_4	$2{\cdot}2 \times 10^8$	$2{\cdot}2 \times 10^8$	$3{\cdot}2 \times 10^8$	$2{\cdot}3 \times 10^8$
M	230	250	110	240
M by static spectrophotometry	330	350	130	240

meter, with the wavelengths and extinction coefficients of Rossi-Fanelli *et al.* (1959).

Kinetic experiments. These were carried out with the stopped-flow apparatus of Gibson (1954), to give the values of k_{43} by the method of Gibson & Roughton (1955), and of l_{01} by the method of Gibson & Roughton (1957*a*). Values of k_{34} and l_{34} were obtained by the method of flash photolysis with the apparatus of Gibson (1959). The procedure for determining l_{34} was that described by Gibson (1956); for k_{34} the method was modified by increasing the flash energy to correspond to the lower photosensitivity of oxyhaemoglobin (Gibson & Ainsworth, 1957), and omitting the step of converting into carboxyhaemoglobin; otherwise the determinations were carried out as for l_{34}. The value of l_{43} was obtained by replacement of CO by NO with the spectrophotometric method of Gibson & Roughton (1957*b*).

Partition of haemoglobin between oxygen and carbon monoxide

When haemoglobin is equilibrated with a mixture of O_2 and CO such that a negligible proportion of gas-binding groups remains free, the partition coefficient

$$M = L_4/K_4 = ([O_2]\cdot[COHb])/([CO]\cdot[O_2Hb]).$$

This coefficient forms a useful check on the kinetic work in which k_{34}, k_{43}, l_{34} and l_{43} were determined, since $K_4 = k_{34}/k_{43}$ and $L_4 = l_{34}/l_{43}$. A value of M is thus obtained from kinetic measurements which can be compared with the value determined directly. The method used was to place a dilute (10 μM) solution of oxyhaemoglobin in a tonometer attached to a spectrophotometer cell and record the absorption spectrum between 380 and 450 mμ (with an Optica recording spectrophotometer, Optica Ltd., Newcastle upon Tyne). The haemoglobin was then equilibrated in the dark with a gas mixture containing CO + O_2 (1:250, v/v), and the spectrum again recorded. The tonometer was finally filled with pure CO and the spectrum of the CO-compound recorded. The three records were made on the same piece of chart, and the extinction was measured for each record at the wavelength of maximum absorption of the CO-compound. The proportions of oxy- and carboxyhaemoglobin in the mixture were calculated assuming Beer's law to apply.

Table 2. *Combination of the first molecule of carbon monoxide with fully reduced haemoglobin*

Reaction velocities were measured by the stopped-flow method. Total pigment concentration after mixing was 5 μM (as haematin iron); concentration of CO, 0·11 mM; phosphate buffer, 0·1 M, pH 7·1; temperature, 20°. Wavelengths: proto-haemoglobin 430 mμ, mesohaemoglobin and deuterohaemoglobin 421 mμ. Values of l_{34} are taken from Table 1.

Haemoglobin	$10^{-5} \times l_{01}$ (M^{-1} sec.$^{-1}$)	Ratio l_{34}/l_{01}
Natural protohaemoglobin	4·4	16
Reconstituted protohaemoglobin	4·8	15
Mesohaemoglobin	11·6	6
Deuterohaemoglobin	7·6	12

RESULTS

The results of experiments on the rate of combination and dissociation of the fourth molecule of oxygen and carbon monoxide are summarized in Table 1. The values are all averages of satisfactory duplicate experiments. It should be noted that in every case the natural protohaemoglobin gave results agreeing within experimental error with those obtained for reconstituted protohaemoglobin. The values agree well with those reported by Gibson, Kreuzer, Meda & Roughton (1955) for human haemoglobin at pH 7·1, although their experiments did not cover so wide a range of velocity constants and were usually obtained with much higher concentrations of haemoglobin. Although the values of M calculated from the kinetic experiments differ appreciably from those directly determined, the possibilities of cumulative error in five independent determinations are such that the agreement is considered satisfactory. Inspection of Table 1 shows that the largest differences between protohaemoglobin and mesohaemoglobin and deuterohaemoglobin are found in the values for k_{43}. The other three kinetic constants determined show much smaller differences.

Table 3. *Comparison of observed and calculated results for the combination of carbon monoxide with reconstituted protohaemoglobin, mesohaemoglobin and deuterohaemoglobin*

In each case l_{34} was taken to be infinitely large. The experimental conditions were those of Table 2.

Time (msec.)	Protohaemoglobin		Mesohaemoglobin		Deuterohaemoglobin	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
5	—	—	85·5	86·9	—	—
10	87·7	87·2	74·2	74·3	81·0	80·7
15	—	—	63·3	62·5	—	—
20	75·8	75·8	53·8	52·9	61·3	62·0
30	64·5	64·6	38·1	37·8	44·8	45·1
40	54·6	54·5	26·9	27·1	31·3	31·5
50	45·7	46·0	19·2	19·0	21·3	21·2
60	37·4	37·5	—	—	14·2	13·8
70	30·7	31·0	—	—	—	—

Ratios used in calculating figures for the Table.

	l_{01}	l_{12}	l_{23}
Protohaemoglobin	4	3	2
Mesohaemoglobin	4	10	1·5
Deuterohaemoglobin	4	3·2	3

Values for the rate of combination of the first molecule of carbon monoxide are given in Table 2. They were obtained by the extrapolation procedure described by Gibson & Roughton (1957*a*). There is no significant difference between natural and reconstituted protohaemoglobins, but both mesohaemoglobin and deuterohaemoglobin show a substantially higher rate of reaction. Comparison of the form of the progress curve of the reaction of carbon monoxide with the three haemoglobins showed striking differences: protohaemoglobin showed a gentle increase in rate as the reaction progressed, which was closely similar to that observed by Gibson & Roughton (1957*a*) with sheep haemoglobin. With deuterohaemoglobin an increase was again observed, which was, however, appreciably sharper than that seen with protohaemoglobin. With mesohaemoglobin little or no increase in rate occurred during the reaction. Gibson & Roughton (1957*a*) have dealt with the problem of evaluating all four combination velocity constants from data of this type. Their full procedure requires extensive experimental information and expert statistical analysis which were not available to us. We have, however, made a rough estimate of the relative sizes of the four constants, by somewhat simpler methods. Thus Table 2 shows that the ratio l_{34}/l_{01} is in every case much greater than the statistical value of 0·25. Gibson & Roughton (1957*a*) have shown that any value of this ratio greater than about 6 may be treated as being infinitely large. Taking the values of l_{01} from Table 2, and l_{34} as ∞, attempts to fit the experimental curves by Desk Method II of Gibson & Roughton (1957*a*) have been made on a trial-and-error basis. The fit achieved is illustrated in Table 3, which gives also the values of the ratios $l_{01}:l_{12}:l_{23}$ used in working out the calculated values. The general result emerges that the ratios for protohaemoglobin and deuterohaemoglobin do not differ widely from each other or from the statistical set of ratios, 4:3:2. Mesohaemoglobin does not fit into this pattern, and reasonable agreement between the observed and calculated values requires that l_{12} shall be considerably above the statistical value and l_{23} below it. The precision with which l_{12} can be estimated is, however, low: it can only be said with any assurance that it must be large by comparison with l_{01} and l_{23}.

DISCUSSION

Natural and reconstituted protohaemoglobins

Rossi-Fanelli & Antonini (1959*a*) have shown that the oxygen-dissociation curves of natural and reconstituted protohaemoglobins are similar, and the kinetic work reported here now extends their findings to include individual kinetic and equilibrium constants. The results suggest strongly that the natural and reconstituted proteins are, within the limits of experimental error, exactly equivalent in their functional properties, and that this equivalence extends even to such an easily disturbed character as haem–haem interaction, which can be profoundly modified by relatively slight changes in the protein molecule. It is difficult to believe that such close similarity of behaviour between the natural and reconstituted haemoglobins could be observed unless the haem groups return, on reconstititution, to positions exactly analogous to those which they occupied in the natural protein. This view is exactly contrary to the conclusion reached by Wyman (1948), on the experimental evidence of Davies. The reasons for

the discrepancy are not clear, but no doubt arise from the three main differences in the experimental procedure: (i) Davies used horse haemoglobin whereas the present work has been carried out with human haemoglobin; (ii) the procedure of Rossi-Fanelli *et al.* (1958) for preparing the apo-protein is carried out under mild conditions; (iii) methaemoglobin has been reduced enzymically instead of with dithionite. It should also be pointed out that the preparation of human haemoglobin used by Rossi-Fanelli *et al.* (1958) has been very fully studied not only in regard to its reversible reactions with ligands, but also in its ultracentrifugal and electrophoretic behaviour, and in its resistance to heat and alkaline denaturation. The close agreement found in every property studied justifies, in our opinion, the belief that the natural and reconstituted haemoglobins are, in fact, identical.

The finding (Table 3) that the first three molecules of carbon monoxide to combine with protohaemoglobin do so at rates in the ratios 4:3:2 is unexpected, since the indications from equilibrium work suggested that a sequence such as 4:10:2 was more likely. The determinations of Lyster (1957) showed that with horse and with human haemoglobins K_2 was considerably greater than K_1, whereas Roughton *et al.* (1955) found with sheep haemoglobin that K_1, K_2 and K_3 were all of the same order. There is no information available about the values of L_1 and L_2, however, for any species of haemoglobin, and very little weight can be attached to speculations grounded on comparisons of kinetic work with carbon monoxide and equilibrium work with oxygen.

Mesohaemoglobin and deuterohaemoglobin

The kinetic results summarized in Tables 1–3 show that the combination of the first molecule of carbon monoxide with fully reduced mesohaemoglobin and deuterohaemoglobin takes place two or three times as fast as with protohaemoglobin. This result is similar to the findings of Smith & Gibson (1959) and Smith (1959) that carbon monoxide combined more rapidly with several derivatives of mesohaem and deuterohaem than with the corresponding protohaem compounds. In their work, however, mesomyoglobin formed an exception, combining with carbon monoxide at the same rate as did protomyoglobin.

The differences between mesohaemoglobin and deuterohaemoglobin and protohaemoglobin do not extend to the combination of the fourth molecule of carbon monoxide, where the rate is substantially similar for all three proteins. The effect of this is that haem–haem interaction, as measured by the ratio l_{34}/l_{01}, is smaller for mesohaemoglobin and deuterohaemoglobin than for protohaemoglobin.

In the properties discussed so far, mesohaemoglobin and deuterohaemoglobin have shown similar behaviour. The analysis of the course of the reaction with carbon monoxide (Table 3), however, indicates considerable differences between them. Thus deuterohaemoglobin shows a pattern of kinetic constants similar to that for protohaemoglobin, where the marked increase in rate of combination is restricted to the reaction $Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$. Mesohaemoglobin, on the other hand, behaves more as if the haem–haem interaction took place between pairs of haems, the second and fourth velocity constants both being relatively large. This finding depends on the analysis of the time course of the combination reaction; although the differences between mesohaemoglobin and deuterohaemoglobin are well outside the limits of experimental error, their sense is such that it would be possible for heterogeneity in the kinetic properties of the mesohaemoglobin preparation to produce the observed result. There is, however, no reason to suppose that the mesohaemoglobin preparation was heterogeneous.

The dissociation velocity constant for carbon monoxide, l_{43}, does not show outstandingly great differences between the three haemoglobins. The corresponding oxygen constant, k_{43}, however, shows a large difference between mesohaemoglobin and deuterohaemoglobin, which differ by seven to eight times in their rate of reaction. This is much the largest difference found and is chiefly responsible for the differences in K_4 and M which distinguish these haemoproteins. There is a lack of correlation between k_{43} and l_{43}, another example of the breakdown of Haldane's second principle [see Wyman's (1948) discussion].

Rossi-Fanelli & Antonini (1959*b*) and Rossi-Fanelli *et al.* (1959) have examined the oxygen-dissociation curves of mesohaemoglobin and deuterohaemoglobin, which showed an increased affinity for oxygen together with a decreased inflexion, n in Hill's (1910) equation being 1·7 as against 2·7 for protohaemoglobin. Unfortunately our data are not sufficient to allow us to discuss the kinetic origins of these equilibrium effects fully. The differences in affinity between mesohaemoglobin and deuterohaemoglobin are, however, in the direction to be expected from the values of k_{43} and k_{34}. Preliminary experiments on the overall oxygen-combination reaction suggest that mesohaemoglobin may be particularly suitable for more detailed study, especially if its reactions can be accounted for in terms of interaction between pairs of haems, rather than between groups of four haems.

The roles of the haem and of the protein in producing the differences between protohaemoglobin, mesohaemoglobin and deuterohaemoglobin cannot, at present, be precisely defined. The results

of Smith (1959) suggest, however, that the combination velocity rates may be determined chiefly by the chemical configuration of the haems, which apparently all combine with the same site on the protein surface (Rossi-Fanelli & Antonini, 1959*b*), though not necessarily forming identical linkages. Determinations have shown that the ultracentrifugal molecular weight is the same in all three cases, so that the lower degree of haem–haem interaction shown by mesohaemoglobin and deuterohaemoglobin cannot be attributed to splitting to half-molecules. The results reported here suggest that experiments with more widely different haems may help in understanding the mechanism of haemoglobin function.

SUMMARY

1. The rates of combination of natural and reconstituted protohaemoglobin with carbon monoxide have been compared both for the first and fourth molecules of carbon monoxide to combine. The natural and reconstituted proteins give rates agreeing with one another.

2. The rates of dissociation of the first molecule of oxygen and of carbon monoxide from fully saturated natural and reconstituted protohaemoglobin have been similarly compared and found to agree.

3. The corresponding reactions of mesohaemoglobin and deuterohaemoglobin have been examined. The rate of combination of the first molecule of carbon monoxide is greater than for protohaemoglobin. The rate of dissociation of the first molecule of oxygen from saturated haemoglobin is slow for mesohaemoglobin (k_{43} 8 sec.$^{-1}$) and rapid for deuterohaemoglobin (k_{43} 60 sec.$^{-1}$).

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REFERENCES

Adair, G. S. (1925). *J. biol. Chem.* **63**, 529.
Erdmann, J. G. & Corwin, A. H. (1947). *J. Amer. chem. Soc.* **69**, 750.
Fischer, H. F. & Orth, H. O. (1940*a*). *Die Chemie des Pyrrols*, vol. 2, part 1, p. 414. Leipzig: Akademische Verlagsgesellschaft.
Fischer, H. F. & Orth, H. O. (1940*b*). *Die Chemie des Pyrrols*, vol. 2, part 1, p. 445. Leipzig: Akademische Verlagsgesellschaft.
Gibson, Q. H. (1954). *Disc. Faraday Soc.* **17**, 137.
Gibson, Q. H. (1956). *J. Physiol.* **134**, 123.
Gibson, Q. H. (1959). *Biochem. J.* **71**, 293.
Gibson, Q. H. & Ainsworth, S. (1957). *Nature, Lond.*, **180**, 1416.
Gibson, Q. H., Kreuzer, F., Meda, E. & Roughton, F. J. W. (1955). *J. Physiol.* **129**, 65.
Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc* B, **143**, 310.
Gibson, Q. H. & Roughton, F. J. W. (1957*a*). *Proc. Roy. Soc.* B, **146**, 206.
Gibson, Q. H. & Roughton, F. J. W. (1957*b*). *Proc. Roy. Soc.* B, **147**, 44.
Hill, A. V. (1910). *J. Physiol.* **40**, iv.
Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers Inc.
Lyster, R. L. J. (1957). Ph.D. Thesis: University of Cambridge.
Rossi-Fanelli, A. & Antonini, E. (1959*a*). *Arch. Biochem. Biophys.* **80**, 299.
Rossi-Fanelli, A. & Antonini, E. (1959*b*). *Arch. Biochem. Biophys.* **80**, 308.
Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958). *Biochim. biophys. Acta*, **30**, 608.
Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1959). *Arch. Biochem. Biophys.* (in the Press).
Roughton, F. J. W. (1949). *Haemoglobin*, p. 83. London: Butterworths Scientific Publications.
Roughton, F. J. W., Otis, A. B. & Lyster, R. L. J. (1955). *Proc. Roy. Soc.* B, **144**, 29.
Smith, M. H. (1959). *Biochem. J.* **73**, 90.
Smith, M. H. & Gibson, Q. H. (1959). *Biochem. J.* **73**, 101.
Wyman, J. (1948). *Advanc. Protein Chem.* **4**, 408.

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Study of a Proteolytic Enzyme from Rabbit Spleen

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It has long been known that aqueous extracts of spleen exhibit proteinase activity (Hedin, 1903). The intracellular enzymes responsible for this activity and a similar activity found in other tissues have been named cathepsins (Willstätter & Bamann, 1929). The cathepsins of ox spleen have been studied in some detail and classified according to their specificity toward synthetic substrates (Tallan, Jones & Fruton, 1952).

In order to study the catabolism of a protein

antigen one of us undertook the investigation of the degradation of human-serum albumin by aqueous extracts of rabbit spleen. It was found that the extracts were capable of causing the degradation of human-serum albumin at pH 3·0–4·0 (Lapresle, 1955*a*). The immunochemical study of the products of this degradation showed that some of these fragments precipitated independently with distinct antibodies present in sera of rabbits previously immunized with undegraded human-serum albumin (Lapresle, 1955*b*). It was concluded that the albumin molecule had different antigenic groups which were part of different split products.

A comparative study of the degradation products obtained by the action of several proteolytic enzymes on human-serum albumin has shown that spleen extract was a convenient tool for studying the antigenic structure of the albumin molecule (Lapresle, Kaminski & Tanner, 1959). Moreover, an enzyme or enzymes of spleen extracts capable of degrading human-serum albumin in this manner could have an important role in the catabolism of proteins. For these two reasons we have undertaken the study of this activity and the partial purification of the enzyme responsible. Preliminary results of these studies have been reported elsewhere (Lapresle & Webb, 1960).

MATERIALS

Rabbit spleens. Rabbit spleens were obtained from exsanguinated rabbits and frozen for storage.

Human-serum albumin. Squibb fraction V was prepared by ethanol precipitation and was kindly given by the American Red Cross.

Diethylaminoethylcellulose and carboxymethylcellulose. These were prepared according to the methods described by Peterson & Sober (1956) and were obtained from Brown and Co., 500 Fifth Avenue, New York, 36, U.S.A.

Antisera. Rabbit sera were prepared by intravenous injection of alum-precipitated human-serum albumin as described by Lapresle (1955*b*).

METHODS

Protein determination. The amount of protein in the enzyme preparations was determined, with the biuret reagent prepared according to Weichselbaum (1946), by reference to a standard curve prepared with various weights of a freeze-dried crude spleen extract.

Enzyme assay. A solution of human-serum albumin, 10% in water containing 2 mM-$CaCl_2$, 0·2 mM-$MgCl_2$ and 8 mM-cysteine, was adjusted to pH 3·5 with HCl. Albumin solution (0·5 ml.) was added to 0·5 ml. of enzyme preparation (in 0·15M-NaCl) and the mixture was incubated for 30 min. at 45°. Then 1 ml. of 4% trichloroacetic acid was added and the suspension was incubated for 10 min. at 45° and centrifuged. A portion (1 ml.) of the clear supernatant was added to 1·5 ml. of biuret reagent prepared according to Weichselbaum (1946) but containing twice the usual quantity of NaOH in order to neutralize the trichloroacetic acid. After 30 min. of incubation at 37° the extinction of the colour developed by the biuret reagent was read at 555 mμ. Enzyme–substrate blanks were prepared by incubating the substrate solution for 30 min. at 45° and then adding the enzyme and trichloroacetic acid simultaneously. A curve was drawn relating extinction of the supernatant to milligrams of crude extract used. An arbitrary unit of proteolytic activity was defined from the tangent to the initial part of the curve as the activity which would cause an increase of 0·001 unit of extinction/min. of digestion. A further standard curve (Fig. 1) was then constructed relating the increases in extinction of the supernatant to the number of units employed.

Determination of optimum pH. The same conditions were used as those for the measurement of enzyme activity except that albumin solutions were prepared with the pH adjusted with HCl to 2·5, 3·0, 3·5, 4·0, 4·5 and 5·0 as measured by a Radiometer pH meter.

Determination of the Michaelis constant. The Michaelis constant was determined under the following conditions: pH 3·5, temp. 45°, reaction time 30 min. The velocity of the reaction was measured by the quantity of material formed which was no longer precipitated with 2% trichloroacetic acid. To increasing amounts of human-serum albumin a constant quantity of 'crude' preparation (3·3 units) of 'purified' preparation (3·0 units) was used. The Michaelis constant was calculated by the graphic method of Lineweaver & Burk (1934) relating the reciprocal of the initial velocity of the reaction to the reciprocal of the substrate concentration.

Preparation of the enzyme

Step 1. The frozen rabbit spleens were broken in a small volume of water with a Waring Blendor for 1 min. and dialysed with agitation against distilled water for 1 hr. The suspension was centrifuged at 15 000 *g* for 1 hr. and the clear supernatant was decanted.

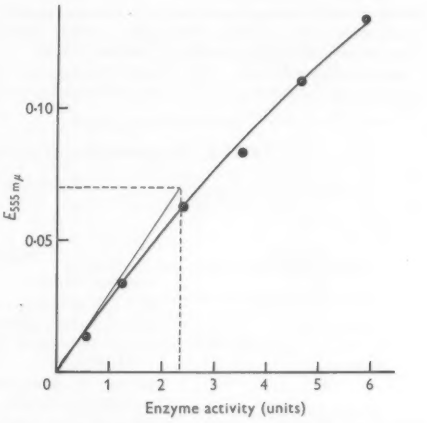

Fig. 1. Standard curve for the digestion of human-serum albumin by rabbit-spleen extract. The extinction figures refer to changes/30 min.

Step II. Sufficient NaCl was added to the aqueous extract to give a concentration of 0·15 M and HCl was added to give pH 5·0. The suspension thus formed was centrifuged and the precipitate was discarded.

Step III. The clear supernatant was precipitated by the addition of 2 vol. of saturated $(NH_4)_2SO_4$ solution, pH 7. After 30 min. the suspension was centrifuged and the supernatant discarded. The precipitate was dissolved in a small volume of water, dialysed free of $(NH_4)_2SO_4$ and frozen for storage. This preparation is referred to as the 'crude' preparation.

Step IV. Further purification was achieved by adsorbing a portion of the inactive material on to diethylaminoethylcellulose equilibrated with a 0·03 M-potassium phosphate buffer, pH 8·0. This adsorption was carried out by mixing the cellulose into a dialysed solution of the enzyme preparation (3 g. of cellulose/g. of protein). After 30 min. of contact the reddish supernatant was filtered from the cellulose and the cellulose washed with 0·5 vol. of phosphate buffer.

Step V. The eluate thus obtained was dialysed against a 15 mM-potassium phosphate buffer, pH 6·75, and mixed with carboxymethylcellulose equilibrated with the same buffer (15 g. of cellulose/g. of protein). After 30 min. of contact the supernatant was again filtered from the cellulose and the cellulose washed with 0·5 vol. of phosphate buffer.

Step VI. The eluate was dialysed against 5 mM-potassium phosphate buffer, pH 8, and put on a column of diethylaminoethylcellulose equilibrated with the same buffer. Some of the inactive material was eliminated by washing the column with the same buffer. The active material was eluted with a 0·1 M-potassium phosphate buffer, pH 7. This preparation is referred to as the 'purified' enzyme.

Chromatographic analysis. The chromatographic analysis was carried out with a preparation obtained at step V. The preparation was dialysed against a 5 mM-potassium phosphate buffer, pH 8, and put on a column of diethylaminoethylcellulose equilibrated with the same buffer. After the passage of a 'break through' the elution of the enzyme was obtained with a concave gradient of potassium phosphate buffer from 5 mM, pH 8, to 0·2 M, pH 7, realized with the cone–sphere technique of Sober & Peterson (1958).

Immunoelectrophoresis. For these experiments the albumin was degraded under the same conditions as for the measurement of proteolytic activity except that the final albumin concentration was 2% and the time allowed 1 hr. The degradation was stopped by neutralizing the reaction medium with one drop of NaOH solution at a suitable concentration. Immunoelectrophoresis of human-serum albumin degraded by the 'crude' and by the 'purified' enzyme was done according to the technique of Grabar & Williams (1955) under the following conditions: veronal–HCl buffer 0·05 M, pH 8·2, duration 4 hr. A rabbit serum anti-human-serum albumin was used to develop the immunoelectrophoresis pattern.

RESULTS

Preparation of the enzyme. Table 1 gives the results of the different steps in the purification of the proteolytic enzyme. With this procedure, where the preparation is made with the double purpose of purifying the activity and recovering as much activity as possible, the final preparation contains about ten times as much activity per milligram as the original preparation and the overall recovery is about 50%. It should be noted that a much better purification can be obtained if one takes at each step the most active portion, and by this means we have obtained a preparation having about 200 units/mg., i.e. 40 times the specific activity of the original preparation.

Chromatographic analysis. Chromatographic analysis of an enzymic preparation obtained at step V shows (Fig. 2) that the proteolytic activity was eluted as a single peak. All the activity was eluted by phosphate concentration of between 0·01 and 0·04 M, pH 8·2–7·0. In other experiments where the pH of the eluting buffer was kept constant at pH 8·0 the activity was also eluted as a single peak between the same concentrations in phosphate (0·01–0·04 M).

The proteins in the preparation were eluted as several peaks, none of which seemed to be enzyme protein. The peak of activity in Fig. 2 and in other experiments under the same conditions appears

Table 1. *Preparation of a proteolytic enzyme from rabbit-spleen extracts*

For details see Methods section.

Step no.	Procedure	Enzyme activity: Total units	Enzyme activity: Units/mg. of protein
I	Aqueous extract	10 000	5·3
II and III	Removal of inactive material precipitated at pH 5 and subsequent precipitation of active material at 66% saturation by $(NH_4)_2SO_4$	6 600	16·5
IV	Adsorption of inactive material on diethylaminoethylcellulose at pH 8	4 950	22·4
V	Adsorption of inactive material on carboxymethylcellulose at pH 6·75	4 800	27·2
VI	Elution from a column of diethylaminoethylcellulose at pH 7	4 800	55

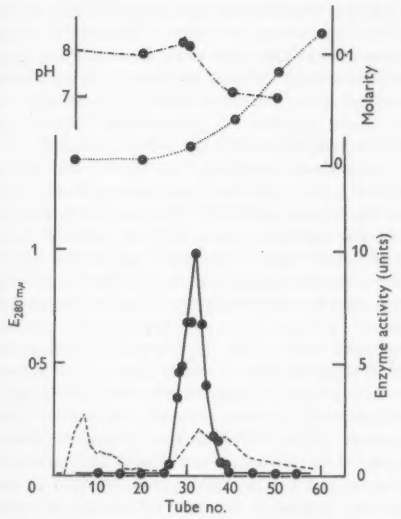

Fig. 2. Chromatography of a purified spleen extract. Enzyme solution (step V; 23 ml.) containing 1·23 mg. of protein/ml. and 21·5 units/ml. was put on a column of 1·5 g. of diethylaminoethylcellulose. The gradient was started at tube no. 19. Each tube contained 5 ml. of eluate. The proteolytic activity of each eluate was measured in duplicate. —, Activity; - - -, extinction.

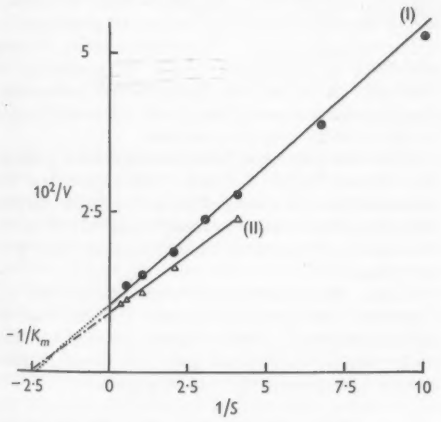

Fig. 3. Graphic representation according to Lineweaver & Burk (1934) for the determination of the Michaelis constant of rabbit-spleen extracts. (I) 'Crude' extract; (II) 'purified' extract. S is the concentration (g./100 ml.) of human-serum albumin used as substrate; V is the extinction at 555 mμ of the colour given by 1 ml. of the trichloroacetic acid supernatants when treated with the biuret reagent.

slightly in advance of the first protein peak eluted by the gradient.

Optimum pH. It was found for both the 'crude' enzyme and the 'purified' enzyme that the activity was optimum over the range pH 3·0–4·5.

Michaelis constant. The study of the enzymic reaction with a constant amount of 'crude' or 'purified' enzyme and increasing amounts of human-serum albumin showed that it obeyed the Michaelis–Menten law. The experimental points thus obtained when plotted according to Lineweaver & Burk corresponded in each case to a straight line (Fig. 3). The Michaelis constant derived from the intercept of these lines with the abscissa was almost the same for the 'crude' enzyme (0·423 g./100 ml.) as for the 'purified' enzyme (0·404 g./100 ml.).

Immunoelectrophoresis. Fig. 4 shows the immunoelectrophoresis of degraded human-serum albumin revealed by an anti-human-serum albumin serum. The split products of human-serum albumin give three different precipitin lines as was described by Lapresle (1955*b*). These lines have the same shape, situation and density whether the albumin has been degraded by a 'crude' or a 'purified' preparation provided that the same number of proteolytic units are used. An example of this pattern is given in Fig. 4 by using human-serum albumin degraded by 10 units/ml. of 'crude' (4 units/mg.) or 'purified' (55 units/mg.) preparation.

DISCUSSION

As we were interested in the enzyme or enzymes of rabbit-spleen extracts which can degrade human-serum albumin, this protein was used as

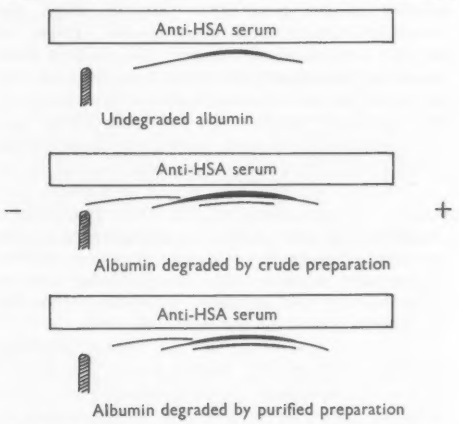

Fig. 4. Schematic representation of the immunoelectrophoresis pattern given by undegraded and degraded human-serum albumin (HSA).

substrate. To measure the activity we arbitrarily used the amount of product formed which is not precipitated in 2 % trichloroacetic acid (the minimum trichloroacetic acid concentration required for complete precipitation of the undegraded human-serum albumin). This non-precipitable material was not measured by its absorption at 280 mμ as is usually done, because the enzyme blanks with the 'crude' preparation were found to contain considerable material which absorbed at this wavelength. The non-precipitable material in the 'crude' enzyme preparation did not react with the biuret reagent. This reagent was therefore used to measure the quantity of the degradation product formed. This procedure is justified by the finding (Lapresle, 1955*a*) that the degraded albumin retains, almost completely, its capacity to react with the biuret reagent. The activity was increased by the presence of cysteine, Ca^{2+} and Mg^{2+} ions, and these substances were added to the reaction medium to avoid the effect of their possible elimination during the course of the purification of the enzyme.

Steps I–IV in the procedure for the preparation of the enzyme are based on preliminary experiments reported by Lapresle & Webb (1960). The main purpose of step V is to remove from the preparation a contaminating reddish pigment which cannot be separated from the activity by chromatography on diethylaminoethylcellulose columns. Step VI eliminates a portion of inactive material as a 'break through' and concentrates the active material.

Chromatographic analysis of the 'crude' preparation carried out by Lapresle & Webb (1960) did not permit a definite conclusion about the homogeneity of the proteolytic enzyme. With the 'stepwise'-elution procedure several peaks of activity were obtained but with the gradient technique one large peak of activity was obtained with an extended and irregular trailing. The analysis of the 'purified' preparation reported herein and several other experiments carried out under similar conditions showed that the protein responsible for the enzymic activity was eluted as a single substance. It would seem that one of the three reasons suggested in our earlier publication (i.e. the existence of a single enzyme in the form of complexes with various other components) was responsible for the apparent existence of several active substances in the 'crude' extracts.

We were interested in the spleen proteases capable of degrading human-serum albumin in such a way as to split the antigenic structure of the albumin molecule in the manner described by Lapresle (1955*b*). Therefore a method has been developed for measuring the proteolytic activity with albumin as substrate, immunoelectrophoresis being used to check the immunochemical properties of the degradation products of albumin. It is most probable that the same activity was being studied by these two procedures because the same immunoelectrophoretic patterns were obtained only when the same number of proteolytic units of each enzyme preparation (steps I–IV) was used.

Under these conditions we have found only one enzyme in the 'purified' preparation (step V). This enzyme is most probably the one which is responsible for the proteolytic activity of the 'crude' preparation since the pH optimum and the Michaelis constant are the same. This conclusion is supported by the similarity of the immunoelectrophoretic patterns given by human-serum albumin degraded with crude and purified preparations, which suggests that in both cases the bonds split were in the same situation relative to the antigenic groups of the albumin molecule. As pointed out by Lapresle *et al.* (1959), human-serum albumin, degraded by pepsin, gives the same immunoelectrophoretic pattern as that given by human-serum albumin degraded by the rabbit-spleen extract, which suggests that the specificity of the rabbit-spleen protease resembles that of pepsin.

The existence of several cathepsins in ox-spleen extracts has been demonstrated by the use of synthetic substrates. The latest classification by this method has permitted the identification of at least three cathepsins: A, B and C (Tallan *et al.* 1952). Lapresle *et al.* (1959) have shown that the enzyme responsible for the degradation of human-serum albumin at pH 3·5 cannot be analogous to either cathepsin B or C. The enzyme which we have described in this work could be analogous to cathepsin A or another intracellular proteinase. Further clarification of this point will await studies on the specificity of this enzyme.

After this paper had been submitted for publication, Press, Porter & Cebra (1960) described the isolation from ox spleen of an intracellular enzyme with properties similar in many respects to those of the enzyme from rabbit spleen that we have been studying.

These two enzymes have the same acid pH optimum, are heat-labile, are eluted from diethylaminoethyl- and carboxymethyl-cellulose under similar conditions and both seem to have specificities resembling that of pepsin.

SUMMARY

1. The proteolytic activity of rabbit-spleen extracts towards human-serum albumin has been studied. With serum albumin as substrate a method of measuring the proteolytic activity of spleen extracts has been developed, based on the determination with the biuret reagent of the

amount of products which are not precipitable by trichloroacetic acid.

2. The enzyme responsible for this proteolytic activity has been purified by adsorption of inactive material on diethylaminoethylcellulose and carboxymethylcellulose.

3. Chromatography on diethylaminoethylcellulose has demonstrated the existence of only one enzyme in the purified preparation.

4. The optimum pH and the Michaelis constant of this enzyme are the same as those of the proteolytic activity in the crude preparation. It was therefore concluded that this enzyme is responsible for the proteolytic activity of the crude spleen extracts.

5. A comparative study of the immunoelectrophoresis patterns given by human-serum albumin, degraded with the crude and purified spleen extracts, has shown that it is this enzyme which is responsible in both cases for the splitting of the antigenic structure of human-serum albumin.

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REFERENCES

Grabar, P. & Williams, C. A., jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
Hedin, S. G. (1903). *J. Physiol.* **30**, 155.
Lapresle, C. (1955*a*). *Bull. Soc. Chim. biol., Paris*, **37**, 969.
Lapresle, C. (1955*b*). *Ann. Inst. Pasteur*, **89**, 654.
Lapresle, C., Kaminski, M. & Tanner, C. E. (1959). *J. Immunol.* **82**, 94.
Lapresle, C. & Webb, T. (1960). *Ciba Foundation Symp., Cellular Aspects of Immunity*. London: J. and A. Churchill Ltd.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Press, E. M., Porter, R. R. & Cebra, J. (1960). *Biochem. J.* **74**, 501.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Tallan, H. H., Jones, M. E. & Fruton, J. S. (1952). *J. biol. Chem.* **194**, 793.
Weichselbaum, T. E. (1946). *Amer. J. clin. Path. tech. Suppl.* **10**, 40.
Willstätter, R. & Bamann, E. (1929). *Hoppe-Seyl. Z.* **180**, 127.

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Effects of the Insecticides DDT and Dieldrin on Phosphorus Metabolism of the Adult Housefly *Musca domestica* L.

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DDT [1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane] and dieldrin (1:2:3:4:10:10-hexachloro-6:7-epoxy-1:4:4*a*:5:6:7:8:8*a*-octahydro-*exo*-1:4-*endo*-5:8-dimethanonaphthalene) respectively represent two important classes of insecticides. Both compounds are toxic to mammals as well as to insects at comparable tissue concentrations, but comparatively little is known of their mode of action in terms of one or more specific biochemical lesions. There is some evidence that DDT may affect oxidative metabolism in a way that is not explained by the exhaustion of endogenous reserves (see review by Winteringham & Lewis, 1959).

The application of a ^{32}P-labelled-pool technique for studying changes in the relative concentrations of the soluble phosphorus compounds of intact adult houseflies as a result of flight, starvation, etc., has been described (Winteringham, 1960). The use of the same technique for studying the effects of DDT and dieldrin is now reported.

EXPERIMENTAL

Treatment and analysis of insect material. Female adult houseflies (*Musca domestica* L.) not less than 3 days old at the time of extraction were used throughout. The strain used was susceptible to the action of DDT or dieldrin. The principal soluble phosphorus compounds were uniformly labelled with ^{32}P *in vivo*, extracted and assayed exactly as explained by Winteringham (1960). Any special treatment of the insects before extraction, such as exposure to insecticides, is indicated in the tables. Insecticides were applied topically to the dorsal surface of the thorax in acetone solution, while the insect was under mild *cyclo*propane anaesthesia. Aqueous glucose was injected intrathoracically by means of an Agla micrometer syringe (Burroughs Wellcome and Co., London) fitted with a 27 s.w.g. needle. Treated insects were always kept at 27–30° in aerated glass chambers without food or water in artificial light. The injection of water alone or the topical application of acetone alone was without detectable effect on phosphorus distribution (Winteringham, 1960).

Tabulation and significance of the data. The results are collected in Tables 1–5. The phosphorus of each compound or fraction (assayed as ^{32}P) is expressed in units % of the total soluble phosphorus recovered from the head (rich in nervous tissue, poor in muscle) or thorax (mainly flight muscle). The significance of differences between the results of single experiments and the means of several experiments has been estimated by the *t* test and corresponding levels of probability (*P*) are indicated. In applying the *t* test it has been assumed that the variance in single experiments with treated insects would be the same as that found in the corresponding groups of control experiments and indicated by the standard deviations (S.D.) of Table 1. A single experiment relates to a group of ten insects.

The description of a fraction in the tables as a particular compound must not be taken to exclude the presence of other unidentified compounds in relatively small but possibly variable proportions. Unresolved composite fractions are indicated by brackets in the tables.

Abbreviations. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), glucose 6-phosphate (G 6-P), arginine phosphoric acid (Arg-P), inorganic phosphate (PO_4), phospholipid recovered under the conditions of extraction (P-lipid). The unidentified compounds UC. 1, UC. 2 and UC. 3 are partly or completely resolved under the routine conditions of paper chromatography used but await identification.

RESULTS

Soluble phosphorus compounds in normal insects. In Table 1 are the mean distributions of soluble phosphorus in the head and thoracic tissues of normal insects which were either active or briefly anaesthetized at the instant of plunging into liquid nitrogen before extraction, etc. The level of thoracic α-glycerophosphate of untreated houseflies was lower when they were active than when they were brought to rest by the use of minimal *cyclo*propane anaesthesia (Winteringham, 1960). *cyclo*Propane was not finally used in some experiments, in which the insects were alive but unable to escape on account of the toxic effects of an insecticide. In comparing the results of treated insects with those of untreated controls account must be taken therefore of whether or not terminal anaesthesia was applied (see Table 1).

Effects of DDT. The results of experiments to determine the soluble phosphorus distribution at different stages of DDT poisoning are compared in Table 2. *cyclo*Propane anaesthesia was applied finally in these experiments. The results in Table 2 should therefore be compared with those of the anaesthetized groups of Table 1.

Changes in phosphorus distribution were apparently related to the condition of the poisoned insects rather than to the time of treatment. For example, the group of insects used in Expt. 1 of Table 2 was evidently less susceptible to the action of DDT than the group used in Expt. 3. Although exposed for a shorter time the insects of Expt. 3 displayed more advanced signs of poisoning and the phosphorus distribution was significantly different (see below) from that of the corresponding control in Table 1. When the insects were 'knocked down' (Expts. 3 and 4, Table 2) the thoracic α-glycerophosphate remained at a significantly low level ($P < 0.05$) despite terminal *cyclo*propane anaesthesia. At the later prostrate stage (Expt. 4, Table 2) there was a significant fall ($P < 0.01$) in thoracic ATP and corresponding accumulation of PO_4 and possibly of AMP.

Insects at the prostrate stage as a result of DDT treatment could sometimes be revived by the injection of aqueous glucose. However, insects revived in this way never recovered the muscular co-ordination of the normal insect. Recovery was associated with a return of ATP and PO_4 towards normal levels in both head and thoracic tissues. The effects of glucose and water injections are shown in Table 3. *cyclo*Propane was not finally applied in these experiments. The results may therefore be compared with those of the un-

Table 1. *Mean soluble phosphorus distribution of the active or anaesthetized housefly*

	Phosphorus of fraction as percentage of the total soluble phosphorus recovered from the head or thorax ± S.D.			
	Normal active insect		Insect lightly anaesthetized	
Fraction	Head (7 expts.)	Thorax (9 expts.)	Head (11 expts.)	Thorax (49 expts.)
ATP + UC. 1	32·0 ± 5·4	32·2 ± 4·4	35·9 ± 6·8	34·7 ± 4·8
ADP	✳5·0 ± 1·8	✳4·7 ± 1·9	✳4·9 ± 2·2	✳3·7 ± 2·4
AMP + UC. 2	4·0 ± 2·3	2·5 ± 1·3	4·3 ± 1·4	3·6 ± 1·9
G 6-P	8·7 ± 2·7	8·3 ± 2·8	10·6 ± 2·8	9·1 ± 3·2
UC. 3	2·0	0·7	0·8	Trace
Arg-P	9·3 ± 6·7	13·4 ± 3·9	6·2 ± 1·7	10·5 ± 3·4
α-Glycerophosphate	13·0 ± 3·6	12·7 ± 3·1	13·0 ± 3·8	20·9 ± 4·6
PO_4	21·7 ± 4·1	20·4 ± 3·8	20·9 ± 4·2	16·8 ± 4·7
P-lipid	4·3	5·1	3·4	0·7
	100	100	100	100

Table 2. *Soluble-phosphorus distributions in adult houseflies at different stages of DDT poisoning*

Expt. 1: 1·0 μg. of DDT was applied topically. Insects were left about 10 hr. without food or water. Insects were hyperactive but still upright. Terminal anaesthesia was applied.

Expt. 2: 1·0 μg. of DDT was applied topically. Insects were left about 5 hr. without food or water. Insects were 'knocked down' and showed unco-ordinated hyperactivity. Terminal anaesthesia was applied.

Expt. 3: 1·0 μg. of DDT was applied topically. Insects were left for 5 hr. without food or water. Insects were definitely 'knocked down' but showed considerable leg activity, etc. Terminal anaesthesia was applied.

Expt. 4: 1·0 μg. of DDT was applied topically. Insects were left 10 hr. without food or water. All were prostrate and showed only feeble movements, tarsal tremors, etc. Terminal anaesthesia was applied.

	Phosphorus of fraction as percentage of the total soluble phosphorus recovered from the head or thorax						
	Expt. 1		Expt. 2		Expt. 3	Expt. 4	
Fraction	Head	Thorax	Head	Thorax	Thorax	Head	Thorax
ATP + UC. 1	28·7	36·2	26·3	30·3	35·9	30·7	14·5
ADP	⁎3·3	⁎4·8	⁎5·0	10·1	0	2·3	⁎1·7
AMP + UC. 2	⌉	⌉	⌉	⌉	1·4	⌉	6·2
G 6-P	22·3	19·0	25·2	16·6	16·1	24·0	6·7
UC. 3	⌋	⌋	⌋	⌋	Trace	⌋	0
Arg-P					12·2		9·2
α-Glycerophosphate	9·7	14·2	10·9	21·1	9·1	8·6	10·5
PO_4	28·2	18·4	32·6	21·9	25·3	34·4	51·2
P-lipid	7·8	7·4	Trace	Trace	Trace	0	Trace
	100	100	100	100	100	100	100

Table 3. *Effects of injecting aqueous glucose into adult houseflies at the prostrate stage of DDT poisoning*

Expt. 1: 1·0 μg. of DDT was applied topically. Insects were left approx. 6 hr. without food or water. All were prostrate but showed slight leg movements. No terminal anaesthesia.

Expt. 2: exactly as in Expt. 1 but 2 μl. of water was injected intrathoracically after 5 hr. Insects showed slight recovery of leg activity after injection of water. Insects were finally extracted 6 hr. after DDT treatment without terminal anaesthesia.

Expt. 3: exactly as in Expt. 1 but 2 μl. of aqueous 10% glucose was injected intrathoracically after 5 hr. Insects showed considerable recovery after injection of glucose but seemed unable to walk properly. Insects were finally extracted 6 hr. after DDT treatment without terminal anaesthesia.

Expt. 4: 1·0 μg. of DDT was applied topically. Insects were left approx. 9 hr. without food or water. All were prostrate but showed slight leg movements. No terminal anaesthesia.

Expt. 5: exactly as in Expt. 4 but 2 μl. of aqueous 10% glucose was injected intrathoracically after 8 hr. All insects showed considerable recovery after injection of glucose but appeared to lack muscular co-ordination. Insects were finally extracted 9 hr. after DDT treatment without terminal anaesthesia.

	Phosphorus of fraction as percentage of the total soluble phosphorus recovered from the head or thorax									
	Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5	
Fraction	Head	Thorax	Head	Thorax	Head	Thorax	Head	Thorax	Head	Thorax
ATP + UC. 1	32·5	26·6	24·5	25·8	33·1	35·7	24·8	20·4	27·2	28·8
ADP	⁎5·0	⁎5·4	⁎6·4	⁎6·9	7·6	⁎3·4	9·3	⁎6·0	12·7	⁎3·9
AMP + UC. 2	⌉	⌉	⌉	⌉	⌉	⌉	⌉	0	⌉	0
G 6-P	19·3	25·4	25·1	25·3	20·7	28·4	20·6	13·9	25·6	19·0
UC. 3	⌋	⌋	⌋	⌋	⌋	⌋	⌋	0	⌋	Trace
Arg-P								9·8		10·5
α-Glycerophosphate	5·6	7·8	6·8	8·5	9·0	6·4	7·2	9·4	6·0	12·0
PO_4	25·9	23·6	29·3	22·1	23·6	21·2	33·4	31·2	23·0	19·3
P-lipid	11·7	11·2	7·9	11·4	6·0	4·9	4·7	9·3	5·5	6·5
	100	100	100	100	100	100	100	100	100	100

anaesthetized groups of Table 1. It will be seen that in both the head and thoracic tissues of the DDT-treated insects the α-glycerophosphate levels were lower than those found in the normal unanaesthetized insects. The results of Expts. 2, 3 and 5 (Table 3) suggest that neither water nor aqueous glucose was effective in restoring α-glycerophosphate to the levels of the normally active insects (Table 1). The results in Table 4 show that DDT-treated insects spared the typical hypermuscular activity by sustained *cyclo*propane anaesthesia are not protected from a significant fall in ATP ($P < 0{\cdot}01$). These results are in agreement with some reported earlier (Winteringham, 1956).

Effects of dieldrin. After the topical application of dieldrin adult houseflies become excited, but, unlike their response to DDT, they do not quickly lose the co-ordination of their legs. Later (1–2 hr. after a 0·37 μg. dose) they enter a stage of 'flight convulsions', during which the wings fan for several seconds at a time with the correct flight frequency but the insects appear to be unable to take off and fly. Sometimes they stand and sometimes they spin round during wing fanning. The insects invariably become 'knocked down' shortly after the stage of flight convulsions and their leg movements progressively become less until a state of complete prostration ensues. No significant

Table 4. *Effects of DDT on the soluble phosphorus distribution of adult houseflies spared hyperactivity by sustained* cyclo*propane anaesthesia*

Expt. 1: insects were deeply anaesthetized by continuous exposure to an air–*cyclo*propane mixture for 3·5 hr. They were finally removed to fresh air for about 30 min. until there were slight movements, to ensure that possibly dead insects were not included.

Expt. 2: exactly as in Expt. 1 but 1·0 μg. of DDT was first applied topically. All insects remained 'knocked down' after the aeration period but were alive. Terminal anaesthesia was not applied.

	Phosphorus of fraction as percentage of the total soluble phosphorus recovered from the head or thorax			
	Expt. 1		Expt. 2	
Fraction	Head	Thorax	Head	Thorax
ATP + UC. 1	37·3	30·6	31·2	20·0
ADP	✳2·2	✳2·0	✳2·9	✳1·1
AMP + UC. 2	┐	┐	┐	┐
G 6-P	27·0	33·0	31·3	34·5
UC. 3	┘	┘	┘	┘
Arg-P				
α-Glycerophosphate	14·4	17·0	8·5	11·3
PO_4	19·1	17·4	26·1	33·1
P-lipid	Trace	Trace	Trace	Trace
	100	100	100	100

Table 5. *Soluble phosphorus distributions in adult houseflies at different stages of dieldrin poisoning*

Expt. 1: 0·37 μg. of dieldrin was applied topically. Insects were left 2 hr. without food or water. They were excited but otherwise normal. Terminal anaesthesia was applied.

Expt. 2: exactly as in Expt. 1 but insects were left 8 hr. All insects were 'knocked down' but showed slight leg movements. Terminal anaesthesia was applied.

Expt. 3: 1·0 μg. of dieldrin was applied topically. Insects were left 2·5 hr. without food or water. All were 'knocked down' but showed considerable leg activity. No terminal anaesthesia.

Expt. 4: exactly as in Expt. 3 but insects were left 24 hr. Only survivors showing slight leg movements were used.

Expt. 5: 0·37 μg. of dieldrin was applied topically. Insects were left 2 hr. without food or water. All were at the stage of 'flight convulsions' and fanning wings at the instant of chilling in liquid nitrogen. Insects were provided with dietary milk for 48 hr. before this experiment.

Expt. 6: exactly as in Expt. 5 but insects were completely immobilized with *cyclo*propane before chilling.

	Phosphorus of fraction as percentage of the total soluble phosphorus recovered from the head or thorax							
	Expt. 1	Expt. 2	Expt. 3		Expt. 4		Expt. 5	Expt. 6
Fraction	Thorax	Thorax	Head	Thorax	Head	Thorax	Thorax	Thorax
ATP + UC. 1	┐	23·4	42·9	30·8	31·0	31·0	42·3	31·7
ADP	37·1 ┘	✳4·1	✳2·8	✳3·0	✳3·9	✳4·6	8·0	9·8
AMP + UC. 2	┐	6·8	5·8	5·5	┐	7·2	0	5·1
G 6-P	23·7	9·8	5·2	7·5	18·2	6·2	5·1	4·6
UC. 3	┘	0	2·3	5·1	┘	6·2	0	0
Arg-P		8·7	3·8	9·6		5·3	5·6	7·8
α-Glycerophosphate	15·3	9·7	8·2	8·0	6·3	6·5	7·9	7·2
PO_4	22·4	32·2	25·2	28·7	33·2	30·3	22·6	15·5
P-lipid	1·5	5·3	3·8	1·8	7·4	2·7	8·5	18·3
	100	100	100	100	100	100	100	100

changes in phosphorus distribution were found until the stage of flight convulsions (see Table 5). At this stage the level of thoracic α-glycerophosphate was as low as or even lower than that of the normal unanaesthetized insect, and, as with DDT poisoning, *cyclo*propane anaesthesia failed to restore the α-glycerophosphate to the resting level (compare Expts. 5 and 6 of Table 5). Instead, it remained significantly low ($P < 0.01$). In one experiment there was a fall in thoracic ATP which was just significant ($P < 0.05$) and a corresponding rise in AMP and PO_4 (Expt. 2, Table 5). If the insects were chilled in liquid nitrogen at the instant of wing fanning during the stage of flight convulsions (Expt. 5, Table 5) there was no evidence of the increase in thoracic PO_4 observed during normal flight (Winteringham, 1960). The results of Table 5 may be compared with those of Table 1.

DISCUSSION

Fall in adenosine triphosphate. It has been reported (Winteringham, 1960) that in houseflies deprived of food and water to the point of prostration there was a significant fall in the ATP of the head tissues and, like the fall in thoracic ATP of DDT-treated insects (see above), this could be reversed by injecting aqueous glucose. Later, when the insect could not be revived by glucose injection, most of the ATP had disappeared from both head and thoracic tissues. The results in Table 4 show that protection of the DDT-treated insect from physical exhaustion by *cyclo*propane anaesthesia failed to prevent a fall in thoracic ATP. It has also been found that *cyclo*propane anaesthesia failed to protect adult houseflies from the enhanced desiccation associated with DDT poisoning. This is shown by the results of Fig. 1. This may also explain why cockroaches exposed to DDT but spared the characteristic hyperactivity and consequent carbohydrate depletion by sustained *cyclo*propane anaesthesia went on to die as though they had not been so protected (Merrill, Savit & Tobias, 1946). Ingram (1955) concluded that the enhanced loss of water by DDT- or by pyrethrum-treated insects was not correlated with hyperactivity or increased respiration. Beard (1958) found ATP depletion in *Galleria* larvae only when prostration was apparently associated with a loss of water by regurgitation.

The accelerated desiccation caused by DDT may also account for the fact reported earlier (Winteringham, 1956) that DDT-induced prostration and the fall in ATP occur before the exhaustion of endogenous reserves as indicated by total oxygen consumption. Heslop & Ray (1959) have similarly observed that prostration of cockroaches exposed to DDT or physical stress occurred when total oxygen uptake indicated only partial depletion of endogenous reserves. Thus the fall in ATP and in rate of oxygen uptake in DDT-poisoned houseflies may be due to loss of the normal ability to draw upon endogenous reserves as a result of desiccation and not due to the direct action of the insecticide.

Levels of α-glycerophosphate. The results of Expts. 3 and 4 of Table 2 and of Expts. 4 and 6 of Table 5 show that in both DDT- and dieldrin-poisoned insects the thoracic α-glycerophosphate remained at a lower level than is found in the normal anaesthetized insect. Moreover, this effect occurred before any significant fall in ATP and, in the dieldrin-poisoned insects, before there was likely to be serious desiccation (Fig. 1).

The simultaneous formation and removal of α-glycerophosphate by a soluble diphosphopyridine nucleotide-dependent dehydrogenase and structurally bound oxidase respectively may provide an important method of transporting hydrogen from the cytoplasm to the respiratory chain of the mitochondria of insect flight muscle (Bücher, Klingenberg & Zebe, 1958; Zebe, Delbrück & Bücher, 1959). The operation of this 'α-glycerophosphate cycle' is indeed consistent with the observed changes in α-glycerophosphate levels at the instant of rest or activity (Winteringham, 1960). Bücher *et al.* (1958) have suggested that *in vivo* the α-glycerophosphate cycle is under respiratory control,

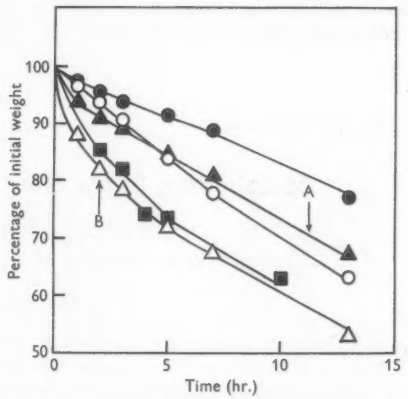

Fig. 1. Loss in weight of groups of 20 adult female houseflies in aerated glass chambers at 27°. No food or water was given. Initial treatments: ●, 1 μl. of acetone; ▲, 2 μl. of acetone; ○, 0·37 μg. of dieldrin/2 μl. of acetone; △, 1 μg. of DDT/1 μl. of acetone; ■, 1 μg. of DDT/1 μl. of acetone and continuous *cyclo*propane anaesthesia. Insects which were exposed to acetone alone behaved normally until they had lost about 30% of their initial weight (A), when signs of complete exhaustion rapidly appeared. All insects that were exposed to DDT or to dieldrin were 'knocked down' within 2 hr. (B).

whereas Sacktor (1958) has postulated a direct control of enzyme activity by means of an endogenous chelating agent. In this context the failure of *cyclo*propane to restore thoracic α-glycerophosphate of the DDT-poisoned insects, and, more especially, of the dieldrin-poisoned insects to their resting levels is of interest. *cyclo*Propane quickly suppresses visible motor activity and, presumably, the increased respiration associated with motor activity (Merrill *et al.* 1946). Yet the α-glycerophosphate remains at a level normally found in the active or flying insect. If the enzymes of the α-glycerophosphate cycle are under nervous control, e.g. through the agency of a neurosecretion or hormone, it is possible that the well-established unstabilization of nervous tissue by DDT or dieldrin is also associated with an interference in the regulation of carbohydrate metabolism at the α-glycerophosphate level. There is some evidence that particulate α-glycerophosphate-oxidase activity may be under thyroid-hormone control (Lee, Takemori & Lardy, 1959).

SUMMARY

1. At the late prostrate stage there is a significant breakdown of thoracic adenosine triphosphate in DDT-poisoned houseflies. This fall could be reversed by injecting aqueous glucose.

2. There was also a significant breakdown of thoracic adenosine triphosphate in DDT-poisoned insects spared the hypermotor activity by *cyclo*propane anaesthesia. *cyclo*Propane also failed to prevent the enhanced desiccation associated with DDT poisoning.

3. The fall in adenosine triphosphate and respiration rate of DDT-poisoned houseflies is not due to the exhaustion of endogenous reserves or to the hypermotor activity induced by DDT.

4. In both DDT- and dieldrin-poisoned houseflies there was a fall in the level of thoracic α-glycerophosphate, which could not be reversed by *cyclo*propane anaesthesia.

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REFERENCES

Beard, R. L. (1958). *Ent. exp. appl.* **1**, 260.
Bücher, T., Klingenberg, M. & Zebe, E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **12**, 153.
Heslop, J. P. & Ray, J. W. (1959). *J. insect. Physiol.* **3**, 395.
Ingram, R. L. (1955). *Ann. ent. Soc. Amer.* **48**, 481.
Lee, Y.-P., Takemori, A. E. & Lardy, H. (1959). *J. biol. Chem.* **234**, 3051.
Merrill, R. S., Savit, J. & Tobias, J. M. (1946). *J. cell. comp. Physiol.* **28**, 465.
Sacktor, B. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **12**, 138.
Winteringham, F. P. W. (1956). *Chem. & Ind.* p. 1182.
Winteringham, F. P. W. (1960). *Biochem. J.* **75**, 38.
Winteringham, F. P. W. & Lewis, S. E. (1959). *Annu. Rev. Ent.* **4**, 303.
Zebe, E., Delbrück, A. & Bücher, T. (1959). *Biochem. Z.* **331**, 254.

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Metabolism in the New Zealand Strain of Obese Mice

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A mutant strain of mice discovered in the Jackson Memorial Laboratory by Ingalls, Dickie & Snell (1950) exhibits the 'hereditary obese-hyperglycaemic syndrome' which has been studied by Mayer and his collaborators (see Mayer, 1953). Guggenheim & Mayer (1952) found that the obese mice liberated $^{14}CO_2$ from [1-^{14}C]acetate at a slower rate than non-obese mice and Hughes & Tolbert (1958) made a similar observation in respect of [2-^{14}C]acetate. Other metabolic anomalies that have been reported in obese as compared with normal mice include increased turnover of hepatic glycogen (Shull & Mayer, 1956) and increased activity of liver phosphorylase (Shull, Ashmore & Mayer, 1956).

The New Zealand strain of obese mice was developed in the Hugh Adam Department of Cancer Research by systematic inbreeding from a pair of mice chosen from a stock colony (Bielschowsky & Bielschowsky, 1953). The New Zealand obese mice exhibit the hyperglycaemic hereditary form of obesity but they differ from the obese strain of the Jackson Memorial Laboratory in several important respects (Bielschowsky & Bielschowsky, 1956).

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The work described in this paper was done with the object of comparing the metabolism of New Zealand obese mice with that of normal mice.

METHODS

Animals. The care of the mice and the composition of the stock diet are described by Bielschowsky & Bielschowsky (1956).

Measurement of respiration

Apparatus. The respirometer was a closed-circuit type of apparatus operated at constant pressure (Fig. 1) in a room which was thermostatically controlled at 24±1° and air-conditioned. The compensator (A) was provided with a stopcock (C) opening to the atmosphere. The compensator was joined to a manometer (B), 40 cm. long (bore 3 mm.), which was half-filled with Brodie's fluid. The volume of air above the water in A was approximately equal to the total volume (400 ml.) of the enclosed air circuit on the other side of the manometer.

The manifold (DEFG) connected the manometer to the air circuit, and, by way of the three-way taps H and K, with the O_2 reservoirs. Tap H communicated with a 50 ml. burette, graduated in 0·1 ml., and tap K with calibrated gas pipettes, one of 150 ml. and the other of 250 ml. capacity, either or both of which could be put in communication with the air circuit. Two T-pieces leading to H and K were provided for attachment of O_2 cylinders. The burette and the gas pipettes were equipped with levelling bulbs which were filled with saturated (at 24°) NaCl solution.

The animal chamber (M) was a cylindrical glass chamber (volume about 150 ml., diam. 5 cm.) consisting of two parts each with a ground-glass flange. The ground surfaces, lubricated with rubber grease to make an air-tight seal, were held together by means of a brass screw-clamp fitted with rubber washers. A stainless-steel wire gauze disk was placed at each end of the interior to prevent the animal from blocking the flow of air. The gas inlet and outlet tubes were provided with bulbs, loosely packed with glass wool, to disperse the air stream that is otherwise discomforting to the animal. The chamber and the gas-leading tubes were immersed in a glass tank of water (about 5 l.) at 24°.

The expired air was dried by passage through a train composed of the bubblers (P and R) containing sulphuric acid (sp.gr. 1·84) and a U-tube (S) three-quarters filled with lumps of dried $CaCl_2$ (analytical reagent) and one-quarter with anhydrone. The CO_2 was trapped in a detachable unit composed of a bubbler containing 10 ml. of carbonate-free 3N-NaOH solution and a guard tube three-quarters filled with soda asbestos and one-quarter with anhydrone. The soda asbestos and the anhydrone (both 14–22 mesh) were microanalytical reagents. Four of these units were connected in parallel but only one was used at a time. The CO_2-absorbing units were followed by a bubbler (T) containing saturated $Ba(OH)_2$ solution to act as a visual check on the absorption of CO_2.

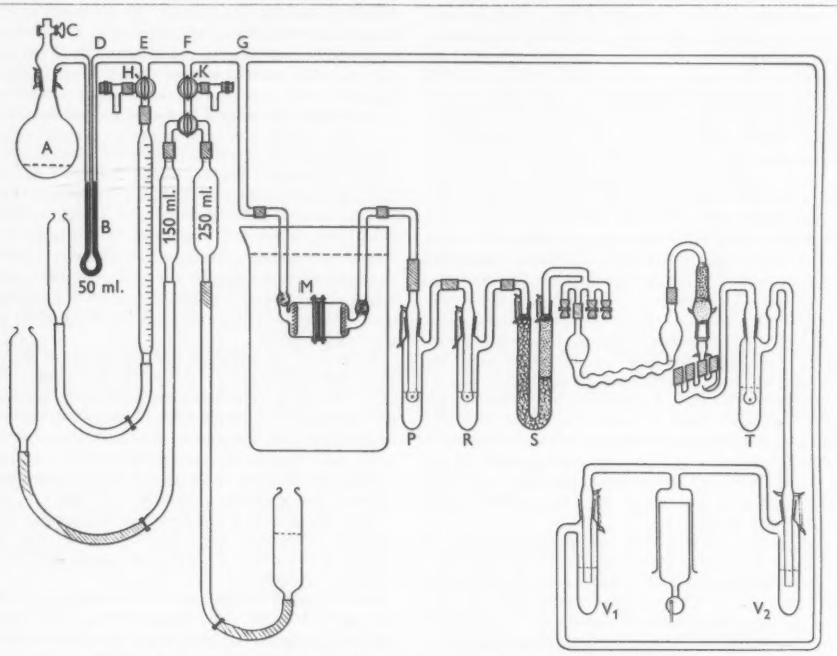

Fig. 1. Closed-circuit apparatus for measurement of respiratory exchange in mice. The lettering is that used in the text.

The air-circulating pump consisted of the bubbler valves (V_1, V_2) filled with saturated (at 24°) NaCl solution, and a 25 ml. graduated glass syringe, the plunger of which was attached by a crank shaft to an eccentric pulley and driven by a geared 0·05 h.p. motor. During an experiment the ground surface of the plunger was kept well lubricated with machine oil. The pump operated at about 60 strokes/min., circulating the air at approximately 1 l./min.

When the apparatus was tested for leaks, under a positive or a negative pressure equal to 7 mm. Hg, with the pump working, the change in volume did not exceed 0·6 ml. in 6 hr. Since no experiment lasted more than 100 min. and the O_2 uptake was not less than 60 ml., the error due to leakage was negligible.

Procedure. The 150 ml. and 250 ml. pipettes were filled to the lower calibration marks with O_2 at atmospheric pressure and the tap K was closed. The burette was filled similarly and tap H closed. In these operations the T-pieces were used to flush the connexions with gas.

The mouse was placed in the animal chamber and the two parts were sealed together. A small mat of filter paper was placed beneath the animal to absorb any urine passed. The chamber was immersed in the tank with the gas inlet joined to the circuit but the gas outlet unattached. The tank was covered with a black cloth and open ventilation started. The mouse was usually active for about 30 min. After it had been at rest continuously for about 10 min. the circuit was closed, the O_2 burette opened and the mouse allowed to become accustomed to the chamber for a further 10 min. The compensator was closed and the barometer read.

The pump was stopped, a tared CO_2-absorbing unit was substituted for the unit used in the preliminary operations, and the O_2 burette read after the manometric levels had been equalized. The position of the head of the plunger in the syringe was read and the pump started. These operations did not take more than 0·5 min. and were always carried out in the same order. O_2 was slowly admitted to the system from the appropriate O_2 pipette (depending on the size of the animal) by manipulating tap K in such a way as to maintain the levels approximately equal on both sides of the manometer. Thus the rate of admission of O_2 was approximately equal to the rate of consumption. When the levelling fluid reached the upper calibration mark, the screw clip on the rubber connexion to the levelling bulb was closed and for a few minutes O_2 was added to the circuit from the burette. The pump was stopped and the plunger adjusted to the initial reading. The manometric levels were equalized by adjusting the levelling-bulb attached to the burette, and the final burette reading was taken. The O_2 consumption was the sum of the calibrated volume of the O_2 pipette and the volume contraction in the burette. The volume of O_2 was corrected to dry gas at s.t.p. Production of CO_2 was determined as the increase in weight of the whole absorbing unit (bubbler and guard tube). Rates of CO_2 formation were determined by using several of the readily interchangeable units.

The respiration of the normal mouse was measured for 60–100 min. and that of the obese mouse for 30–60 min. Duplicate measurements in the resting mouse did not deviate by more than ±1%. Water did not condense on the inner walls of the chamber at any time during the experiments. Dissipation of body heat appears to have been satisfactory since there was no detectable change in the internal temperature of the chamber.

Studies with ^{14}C-labelled compounds. In experiments involving the incorporation of ^{14}C into expired CO_2 the mouse was injected intraperitoneally with 0·2±0·002 ml. of a solution of the ^{14}C-labelled compound (0·1 m-mole containing approximately 1·0 μc) immediately before it was placed in the animal chamber. In these experiments the preliminary open ventilation period was omitted and the collection of expired CO_2 began within 2 min. The compounds used ([1-^{14}C]glucose, [generally labelled (G)-^{14}C]-glucose, [1-^{14}C]- and [2-^{14}C]-sodium acetate) were obtained from The Radiochemical Centre, Amersham, Bucks, England. The solutions of [^{14}C]acetate were sterilized by steaming in sealed tubes and those of [^{14}C]glucose by filtration.

Analytical methods

Determination of glycogen and fatty acids in the carcass and liver. After removal of the liver the weighed carcass was quickly chopped into pieces about the size of a pea and the whole transferred to 60% (w/v) KOH solution (2ml./g. of carcass) which was heated under reflux for 5 hr. The digest was diluted with 2 vol. of water and decanted from the inorganic residue of the skeleton. Glycogen was precipitated by addition of 1·5 vol. of ethanol, allowed to stand for 4 hr., separated by centrifuging and dissolved in 50 ml. of water. A small portion of the solution was reserved for estimation of total glycogen. The remainder was treated with 2 vol. of ethanol to reprecipitate the glycogen, which was separated by centrifuging and redissolved in 10 ml. of 10% (w/v) trichloroacetic acid. The solution was decolorized with activated charcoal and filtered. The glycogen in the clear filtrate was precipitated by addition of 4 vol. of ethanol, separated by centrifuging and dissolved in 15 ml. of water. Any insoluble material was removed by centrifuging. Precipitation with ethanol was repeated, the product washed successively with ethanol and acetone and dried *in vacuo* over P_2O_5. It was free from N_2 and gave about 95% of reducing substance (calculated as glucose) on acid hydrolysis.

The supernatant liquid obtained from the first precipitation of glycogen contained the potassium salts of fatty acids and the unsaponifiable lipids. It was brought to pH 4 with 10 N-H_2SO_4, extracted repeatedly with light petroleum (b.p. 40–60°) and the extract washed with water. The extract was treated with anhydrous Na_2SO_4, the light petroleum removed and the residue dried to constant weight *in vacuo* over P_2O_5 to give the weight of the fatty acids and unsaponifiable lipids.

With liver the digestion was performed in 30% (w/w) KOH solution (3 ml./g. of liver) heated under reflux for 3 hr. The same procedure was adopted in purifying the glycogen except that the use of decolorizing charcoal was unnecessary. The fatty acids were isolated and determined in the same way as the carcass fatty acids.

Glycogen. This was estimated by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950).

Body nitrogen. This was determined by a macro-Kjeldahl incineration of mice approximately of the same age and weight as those used for the experiments involving radioactive compounds.

Combustions. Combustions of radioactive glycogen (15 mg.) and fatty acids (about 10 mg.) were carried out with the reagents of Van Slyke & Folch (1940) in the apparatus described by Sakami (1955) and the $^{14}CO_2$ was trapped in carbonate-free 3 N-NaOH solution.

Table 1. *Respiration of normal (NZC) and obese mice (NZO) after 3, 6 and 16 hr. of starving*

NZC mice: eight males, six females; mean weight (±S.D.), 30·8±5·0 g.; range 23·4–39·1 g. NZO mice: seven males, eight females; mean weight (±S.D.), 50·0±10·3 g.; range 35·7–66·0 g. The age of animals was 5·0–8·5 months. Temp. of respirometer was 24°. Values given are the means ±S.E.M. (range in parentheses) for single observations on 14 NZC and 15 NZO mice.

Basis of comparison	Period of starvation (hr.)	NZC	NZO	Significance of difference between means (*t* test) *P*
		Oxygen consumption (m-moles/g./hr.)		
Weight	3	0·151±0·0022 (0·130–0·167)	0·162±0·0030 (0·144–0·185)	<0·02≃0·01
	6	0·162±0·0021 (0·149–0·172)	0·172±0·0030 (0·160–0·194)	<0·02>0·01
	16	0·143±0·0023 (0·124–0·155)	0·172±0·0021 (0·159–0·188)	<0·001
		Oxygen consumption (m-moles/g.$^{0·7}$/hr.)		
(Weight)$^{0·7}$	3	0·420±0·011 (0·349–0·485)	0·522±0·009 (0·476–0·604)	<0·001
	6	0·445±0·011 (0·338–0·490)	0·554±0·014 (0·466–0·641)	<0·001
	16	0·399±0·010 (0·333–0·454)	0·557±0·009 (0·507–0·633)	<0·001
		Respiratory quotient		
	3	0·73±0·006 (0·70–0·77)	0·73±0·005 (0·71–0·78)	—
	6	0·72±0·006 (0·69–0·78)	0·72±0·004 (0·70–0·75)	—
	16	0·73±0·006 (0·69–0·78)	0·72±0·006 (0·69–0·77)	—

Measurement of radioactivity. All samples of $^{14}CO_2$ were plated in triplicate on filter paper as $Ba^{14}CO_3$ and counted under a thin end-window Geiger–Müller counter. At least 5000 counts were recorded for each plate. The counts were corrected for background and self-absorption (to infinite thinness). The specific activities of the plates in the triplicate set differed from the mean by less than ±5%.

RESULTS

Respiration of normal and obese mice

The oxygen consumption and carbon dioxide production of fourteen normal strain (NZC) and fifteen mice of New Zealand obese strain (NZO) were determined after withdrawal of food, but not water, for 3, 6 and 16 hr. Each mouse was returned to the stock diet for 3–7 days between the determinations. Table 1 shows a comparison of respiratory rates on the basis of body weight and body surface [(weight)$^{0·7}$]. This has been done because the problems of correlating respiratory activity with body size are not fully resolved (Brody, 1945). These problems include the assessment of fat-free body weight and its use as a basis for comparison. In this connexion it is to be noted that the total lipid content of the NZO mouse is about 38% of the body weight (Bielschowsky & Bielschowsky, 1956) as against 13% for the normal mouse; and the nitrogen content of the NZO mouse is about 1·7% of the body weight as against 2·3% for the NZC mouse. The nitrogen figures represent a small number of determinations. NZC mice, weighing 35·6 and 32·8 g., contained 22·8 and 24·1 mg. of nitrogen/g. respectively; the corresponding figures for NZO mice, weighing 60·4 and 55·8 g., were 17·1 and 17·9 mg. of nitrogen/g. In view of these findings and of the known activity of adipose tissue (Wertheimer & Shapiro, 1948) no attempt has been made to relate respiration to fat-free body weight. Irrespective of the basis of comparison used, the mean oxygen consumption of NZO mice is significantly greater than that of NZC mice after all periods of fasting (Table 1).

Table 2. *Oxygen consumption of mice from the Jackson Memorial Laboratory*

The oxygen consumption of mice starved for 16 hr. has been re-calculated from the data of Mayer, Russell, Bates & Dickie (1952). Compare Table 1. Values are given as means ±S.D.

	Normal mice	Obese mice
Oxygen consumption		
(m-moles/g./hr.)	0·165±0·032	0·074±0·003
(m-moles/g.$^{0·7}$/hr.)	0·469±0·083	0·263±0·008
Weight (g.)	28·2±1·9	53·7±9·1

Table 2 gives the mean oxygen consumption of normal and obese mice from the Jackson Memorial Laboratory for comparison with that of NZC and NZO mice (Table 1, fasted 16 hr.). The strains of normal mice from both Laboratories consume oxygen at approximately the same rate, but the difference between the two strains of obese mice is striking. Whereas the obese mice of the Jackson Memorial Laboratory use oxygen at a slower rate than their normal controls (56% less, computed on the basis of body weight), the NZO mice consume oxygen at a faster rate than NZC mice (20% greater on the basis of body weight).

The R.Q. values for NZO and NZC mice are not significantly different and they are not affected significantly by the period of starvation; but the

mean rate of oxygen consumption of NZC mice is significantly lower ($P < 0.001$) after 16 hr. than after 6 hr. of starvation, whereas that of NZO mice is unchanged (Table 1). In both strains of mice the mean oxygen consumption/g. is somewhat greater after 6 hr. than after 3 hr. of starvation (NZC strain, $P < 0.001$; NZO strain, $P < 0.05 > 0.02$). This trend, which was consistent in all the mice examined, suggests that some adjustment of metabolism may occur between the third and sixth hours of starving.

None of the obese mice used in this work showed glycosuria or ketonuria. The absence of these conditions is characteristic of NZO mice in the age group used.

Oxidation of [^{14}C]acetate and [^{14}C]glucose

The oxygen consumption and carbon dioxide output of NZO and NZC mice, starved for 16 hr., were measured immediately after intraperitoneal injection of 0·1 m-mole of [^{14}C]acetate or [^{14}C]glucose. The carbon dioxide absorber was renewed every 0·5 hr. during the first 2 hr. and later at hourly intervals. The collection of carbon dioxide began a few minutes after the injection, the period of adjustment of the animal to its surroundings being omitted (see Methods section). Results of typical experiments are shown in Table 3, and the numbers of animals used in the whole series of experiments are given in Table 4.

The specific activity of $^{14}CO_2$ formed by both strains of mice after injection of [1-^{14}C]- or [2-^{14}C]-acetate is greatest in the first sample (0–0·5 hr.) and diminishes rapidly in the course of 3 hr. On the other hand, the maximum specific activity is obtained in the second sample (0·5–1·0 hr.) after injection of [1-^{14}C]- or [G-^{14}C]-glucose. The same effect is seen in fed mice after injection of [G-^{14}C]-glucose. The rates of change in the specific activity of $^{14}CO_2$ expired by NZC and NZO mice after administration of all these substances conform to patterns which have been observed in other normal mice (Siegel & Hughes, 1956) and rats (Gould, Sinex, Rosenberg, Solomon & Hastings, 1949) and

Table 3. *Specific activity of $^{14}CO_2$ formed by obese (NZO) and normal (NZC) mice after administration of ^{14}C-labelled compounds*

The ^{14}C-labelled compounds (0·2 ml. of solution containing 0·1 m-mole) were injected intraperitoneally. Temp. of respirometer was 24°. The labelled compounds used (with specific activities in 10^6 counts/min./m-mole) were: Expt. 1, [1-^{14}C]acetate, 1·06; Expt. 2, [2-^{14}C]acetate, 0·9; Expt. 3, [1-^{14}C]glucose, 1·02; Expt. 4, [G-^{14}C]glucose, 1·73. The mice were starved for 16 hr. in Expts. 1–4 but were fed in Expt. 5.

Weight of mouse (g.)			CO_2 produced			
			Amount (m-moles)		Specific activity (counts/min./m-mole)	
NZC	NZO	Time (hr.)	NZC	NZO	NZC	NZO
33·0	59·5	0–0·5	2·38	3·36	30 300	20 900
		0·5–1·0	2·32	2·94	8 070	7 160
		1·0–1·5	2·34	3·25	1 350	1 880
		1·5–2·0	2·61	3·30	570	730
		2·0–3·0	5·46	6·51	270	430
33·0	63·0	0–0·5	2·27	4·02	20 170	11 000
		0·5–1·0	2·10	3·72	10 840	5 290
		1·0–1·5	1·94	3·91	3 500	1 890
		1·5–2·0	1·95	3·93	1 660	950
		2·0–3·0	3·84	7·68	560	390
30·6	58·2	0–0·5	2·16	3·41	7 010	2 410
		0·5–1·0	2·01	3·40	12 200	4 290
		1·0–1·5	1·99	3·18	7 350	3 060
		1·5–2·0	2·04	3·16	4 610	2 610
		2·0–3·0	4·01	6·28	2 110	1 520
29·0	53·0	0–0·5	2·61	3·91	4 510	3 020
		0·5–1·0	2·16	3·00	9 110	5 750
		1·0–1·5	1·88	2·88	6 970	4 450
		1·5–2·0	1·70	2·54	4 420	2 500
		2·0–3·0	3·53	6·10	2 780	1 350
		3·0–4·0	3·58	5·78	1 790	690
		4·0–5·0	3·91	5·10	1 090	410
31·0	58·0	0–0·5	2·05	3·68	8 290	4 760
		0·5–1·0	1·75	3·31	12 280	5 470
		1·0–1·5	1·95	3·24	7 440	4 170
		1·5–2·0	1·77	2·97	6 030	2 510
		2·0–3·0	3·54	6·04	2 530	1 970

in the normal and obese mice from the Jackson Memorial Laboratory (Guggenheim & Mayer, 1952). Since NZO mice have a substantially larger $^{12}CO_2$ output than normal mice, they should expire $^{14}CO_2$ with a specific activity lower than that of $^{14}CO_2$ expired by NZC mice. This expectation is realized (Table 3), but other factors, e.g. differences in substrate pool size, may affect the degree of dilution of $^{14}CO_2$.

Table 4 shows the mean cumulative excretion of $^{14}CO_2$ after the administration of [^{14}C]acetate and [^{14}C]glucose to starved (16 hr.) NZC and NZO mice. In 3 hr. about 90 % of the ^{14}C administered as [1-^{14}C]- or [2-^{14}C]-acetate is eliminated as $^{14}CO_2$ and there is no significant difference between the responses of the normal and the obese mice. Fig. 2 shows that the NZC and NZO mice expire $^{14}CO_2$ derived from [2-^{14}C]acetate at rates which are not significantly different. Essentially the same result was obtained with [1-^{14}C]acetate; but, in accordance with the usual finding in [1-^{14}C]acetate metabolism (Weinman, Strisower & Chaikoff, 1957), the initial rate of $^{14}CO_2$ formation was somewhat faster than with [2-^{14}C]acetate.

Similar experiments with [^{14}C]glucose revealed a different situation (Table 4). With NZC mice the mean recoveries of ^{14}C as $^{14}CO_2$ formed during a 3 hr. period from [1-^{14}C]glucose and [G-^{14}C]glucose were about 68 and 66 % respectively. These figures agree closely with those found by Siegel & Hughes (1956) in their normal mice. Under the same conditions the NZO mice gave recoveries which were about 56 and 52 % of the ^{14}C administered as [1-^{14}C]- and [G-^{14}C]-glucose respectively. This difference between the normal and obese strains is highly significant (Table 4). The difference in the rate of elimination of $^{14}CO_2$ formed from [G-^{14}C]glucose persists throughout a 5 hr. period (Fig. 3) and occurs in fed as well as in starved mice (Table 4). Almost identical curves were obtained after injection

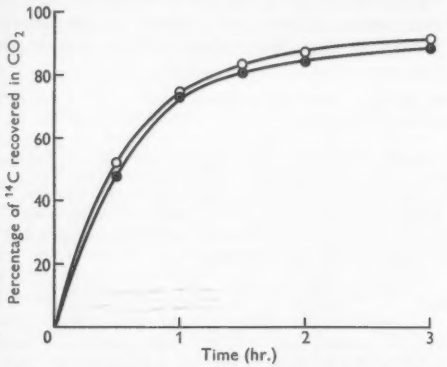

Fig. 2. Cumulative excretion of ^{14}C in expired CO_2 after injection of [2-^{14}C]acetate (0·1 m-mole containing 90 000 counts/min.). The curves represent the mean percentage of ^{14}C recovered in single experiments with eight NZO mice (S.D. ±3·4 %) and eight NZC mice (S.D. ±3·6 %). ○, NZO; ●, NZC.

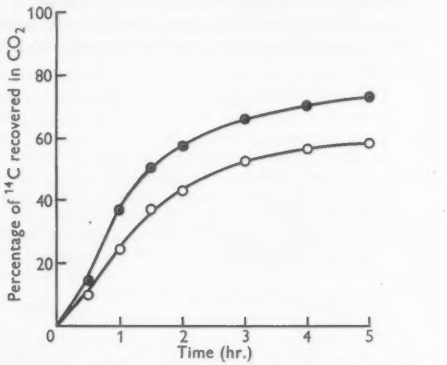

Fig. 3. Cumulative excretion of ^{14}C in expired CO_2 after injection of [G-^{14}C]glucose (0·1 m-mole containing 103 700 counts/min.). The curves represent the mean percentage of ^{14}C recovered in single experiments with eight NZO mice (S.D. ±4·6 %) and eight NZC mice (S.D. ±4·4 %). ○, NZO; ●, NZC.

Table 4. *Cumulative excretion of $^{14}CO_2$ by obese (NZO) and normal (NZC) mice after administration of ^{14}C-labelled compounds*

The ^{14}C-labelled compounds (0·2 ml. of solution containing 0·1 m-mole) were injected intraperitoneally. The mice were 5–9 months old; there were approximately equal numbers of males and females in each group.

No. of mice of each strain	Condition	Substance injected	Total radioactivity (counts/min.)	Mean percentage (±S.E.M.) of ^{14}C recovered as $^{14}CO_2$ in 3 hr. NZC	Mean percentage (±S.E.M.) of ^{14}C recovered as $^{14}CO_2$ in 3 hr. NZO	Significance of difference of the means (*t* test) *P*
8	Starved for 16 hr.	[1-^{14}C]Acetate	106 000	91·6±0·9	94·1±1·1	>0·1
8	Starved for 16 hr.	[2-^{14}C]Acetate	90 000	87·8±1·2	90·4±0·8	>0·1
10	Starved for 16 hr.	[1-^{14}C]Glucose	102 000	68·1±1·5	56·4±1·5	<0·001
8	Starved for 16 hr.	[G-^{14}C]Glucose	104 000	65·8±2·0	51·7±1·3	<0·001
5	Fed	[G-^{14}C]Glucose	104 000	72·0±2·8	61·9±1·6	<0·05>0·02

of [1-^{14}C]glucose, showing that there are no significant differences in the rates of $^{14}CO_2$ formation from the two isotopic forms of glucose.

Incorporation of ^{14}C into glycogen and fatty acids

In 2 hr. NZC and NZO mice in the fed state oxidize about 62 and 54% respectively of the [G-^{14}C]glucose carbon atoms administered. Other transformations of [^{14}C]glucose were studied by isolating glycogen and fatty acids from both liver and carcass. The material designated 'fatty acids' was not treated to remove non-saponifiable lipids. The animals were allowed free access to food and were given an intraperitoneal injection of non-isotopic glucose (50 mg. in 0·2 ml. of solution) 45 min. before [G-^{14}C]glucose was injected by the same route. The animals were killed 2 hr. after administration of the [G-^{14}C]glucose. The results of experiments on five normal and five obese mice are shown in Tables 5 and 6.

It will be seen that the mean weight of the livers of NZO mice is approximately twice that of NZC mice. The hepatic glycogen is distinctly lower in NZO (mean 1·9 ± 0·2% of the liver weight) than in NZC mice (mean 3·6 ± 0·7% of the liver weight), whereas the fatty acid content of the liver is distinctly greater in the obese strain (mean 7·2 ± 2·0% as against 4·7 ± 1·0%). The mean weight of the carcass fatty acids in the obese strain is about four times that in the normal strain; and the mean weight of the carcass glycogen, though variable, is about the same in both strains of mice.

Glycogenesis. The mean recovery of ^{14}C as glycogen was about 5·7% of the dose in the controls and about 2·5% in the obese mice. This difference is largely due to the higher specific activity of the hepatic glycogen formed by the normal strain (Table 5). When the percentages of ^{14}C incorporated/100 mg. of glycogen are compared in the two strains, it will be seen that the liver glycogen of the NZC mice contains nearly five times the amount of ^{14}C found in that of the obese strain (Table 6). With the carcass glycogen the difference between the means is barely significant ($P < 0·05 > 0·02$).

Lipogenesis from glucose. Table 5 shows that the incorporation of ^{14}C derived from glucose into the fatty acids of liver and carcass is substantially greater in the obese (about 9·7%) than in the

Table 5. *Incorporation of ^{14}C into the glycogen and fatty acids of liver and carcass in normal and obese mice*

Five NZC mice and five NZO mice, all males aged 5–6 months, were used. Mean weights (±S.D.) were: NZC mice, 28·0±3·3 g.; NZO mice, 51·6±2·4 g. Mean weights (±S.D.) of livers were: NZC, 0·89±0·11 g.; NZO, 1·99±0·13 g. The animals were given 50 mg. of inert glucose and 0·25 m-mole of [G-^{14}C]glucose (specific activity, 344 000 counts/min./m-mole) by intraperitoneal injection (0·2 ml. of solution) 2 hr. 45 min. and 2 hr. respectively before they were killed. Total radioactivity injected was 516 000 counts/min. No attempt was made to separate non-saponifiable lipids from the material designated 'fatty acids' (see Methods section).

Strain of mice	Material isolated	Mean wt. (±S.D.) (mg.)	Mean specific activity (±S.D.) (counts/min./m-mole)	^{14}C incorporated (mean percentage ±S.D. of the dose)
NZC	Liver glycogen	32·3±8·1	11 750±1650	2·5±0·7
	Carcass glycogen	80·1±29·6	6 770±3200	3·2±1·4
	Liver fatty acids	41·7±7·4	610±240	0·28±0·08
	Carcass fatty acids	3680±1070	86±39	3·4±0·7
NZO	Liver glycogen	35·6±4·7	2 440±990	0·55±0·14
	Carcass glycogen	76·5±21·1	3 590±320	1·9±0·7
	Liver fatty acids	141·7±34·7	390±160	0·62±0·05
	Carcass fatty acids	14 690±1920	55±18	9·1±2·2

Table 6. *Distribution of ^{14}C between the fatty acids and glycogen of liver and carcass in normal and obese mice*

Derived from the data of Table 5.

	Mean percentage (±S.D.) of the dose of ^{14}C incorporated/100 mg. of material isolated			
	Liver		Carcass	
Strain of mice	Fatty acids	Glycogen	Fatty acids	Glycogen
NZC	0·71±0·27	7·8±1·4	0·11±0·06	4·3±2·1
NZO	0·47±0·16	1·6±0·6	0·06±0·03	2·3±0·3
Significance of difference of the means (P)	<0·05>0·02	<0·001	<0·05>0·02	<0·05>0·02

normal mice (about 3·7 %). This appears to be due mainly to the difference in body-fat content. Owing to greater dilution, the fatty acid ^{14}C of the carcass has a lower specific activity than that of the liver in both NZC and NZO mice; but comparisons between the mean specific activities of the fatty acid ^{14}C obtained from the liver, or from the carcass, show that the differences between the two strains of mice are barely significant ($P < 0{\cdot}05 > 0{\cdot}02$). Similarly, the differences between the means are barely significant when the incorporation of ^{14}C is expressed as a percentage of the dose/100 mg. of fatty acids (Table 6).

DISCUSSION

The NZO mouse presents the picture of a 'hereditary obese-hyperglycaemic syndrome'. Various facts described by Bielschowsky & Bielschowsky (1956) point to defective hormonal regulation of carbohydrate and fat metabolism. For example, adult animals show evidence of hyperinsulinism and are resistant to exogenous insulin; and stilboestrol lowers the elevated blood sugar and prevents excessive deposition of body fat in young animals.

The metabolic changes responsible for obesity in NZO mice are likely to be quantitative rather than qualitative. On the basis of comparison used (Table 1), it has been found that the obese strain has a greater respiratory exchange after starving than a control strain of normal mice (NZC). This observation, which is the opposite of the finding in the hyperglycaemic-obese mice of the Jackson Memorial Laboratory (Mayer, Russell, Bates & Dickie, 1952), may be related to the apparently greater activity of the New Zealand strain, which is also distinguished by its fertility and other characteristics (Bielschowsky & Bielschowsky, 1956).

The suggestion that a depressed rate of acetate oxidation may signify abnormal lipogenesis and be a factor in the causation of the obese syndrome (Guggenheim & Mayer, 1952) is not applicable to NZO mice since these animals oxidize [^{14}C]acetate at approximately the same rate as NZC mice (Fig. 2). On the other hand, NZO mice, either fed or starved, eliminate $^{14}CO_2$ derived from [^{14}C]-glucose at a significantly lower rate (about 12 %) than the normal controls. This suggests the possibility that increased lipogenesis from carbohydrate may contribute to the development of the obese condition.

The experiments on the metabolism of [G-^{14}C]-glucose are consistent with this possibility (Table 5). The incorporation of ^{14}C into the liver glycogen of NZO mice (about 0·6 %) is substantially less than that observed in NZC mice (about 2·5 %). The difference, which depends on the higher specific activity of the glycogen formed by the liver of the NZC mouse, could be due to a slower rate of glycogenesis in the obese strain.

The total recovery of ^{14}C given as [G-^{14}C]glucose in the glycogen and fatty acids of the liver and carcass was about 9·5 % in the normal strain and about 12·2 % in the obese strain. The fraction of the dose of ^{14}C that is incorporated into the 'fatty acids' is considerably greater in the obese mice and is roughly proportional to the mass of body fat (Table 5); but when the lipogenic activity of the tissues is expressed as a percentage incorporation of the dose of ^{14}C/100 mg. of fatty acids, the difference between the two strains of mice is merely on the verge of statistical significance (Table 6). If real, this difference might provide a mechanism for the genesis of the obese state and an explanation for a slower rate of glucose oxidation in the NZO mice. An investigation of isolated tissues is desirable.

The comparisons between the NZO and NZC mice were made after injection of the same amount of a ^{14}C-labelled compound into all animals irrespective of body weight. If the rate of equilibration of ^{14}C with body carbon is fast compared with the rate of oxidation of the injected ^{14}C-labelled compound, it would be expected that a lower percentage of ^{14}C would appear in the carbon dioxide expired by the animal of larger body weight. Thus it is possible that difference in body weight alone could explain the fact that NZO mice eliminate $^{14}CO_2$ derived from [^{14}C]glucose at a slower rate than NZC mice. On the other hand, the big difference in body composition may be sufficient to invalidate this argument. The NZO mouse contains only about 27 % more nitrogen (assumed to represent 'active tissue') than the NZC mouse although its body weight is about 70 % greater. For this reason an appropriate fixed dose of ^{14}C (cf. Hughes & Tolbert, 1958) was chosen in preference to a dose proportional to body weight. Differences in body size and composition alone would be expected to affect the specific activities of glycogen and lipids by dilution of ^{14}C; and since ^{14}C is eliminated continuously from the body, the specific activities of these body constituents will also be affected by unknown turnover rates. The extent to which the observed specific activities (Table 5) are influenced by difference in body size or by difference in metabolic behaviour is uncertain.

The differences between the metabolism of the New Zealand strain of obese mice and that of the obese mice from the Jackson Memorial Laboratory serve to emphasize the opinion of Bielschowsky & Bielschowsky (1956) that the 'hereditary hyperglycaemic-obese syndrome' in rodents is not a single entity.

SUMMARY

1. A closed-circuit respirometer has been devised for measurement of the respiratory exchange in mice. It is particularly suitable for ^{14}C-tracer experiments.

2. In the post-absorptive state the New Zealand strain of obese mice has a greater oxygen consumption and carbon dioxide production/g. body weight than a control strain of normal mice.

3. New Zealand obese mice oxidize [1-^{14}C]- or [2-^{14}C]-acetate at approximately the same rate as the control mice. The rates of oxidation of both [G-^{14}C]- and [1-^{14}C]-glucose are about 12% lower in the obese than in the normal strain.

4. The incorporation of ^{14}C derived from [G-^{14}C]-glucose into the glycogen and fatty acids of liver and carcass was determined in New Zealand obese and control mice. Incorporation of ^{14}C into the liver glycogen, but not the carcass glycogen, was considerably less in obese than in normal mice.

5. In several respects the metabolism of the New Zealand strain of obese mice differs from that of the obese strain of the Jackson Memorial Laboratory.

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REFERENCES

Bielschowsky, M. & Bielschowsky, F. (1953). *Proc. Univ. Otago med. Sch.* **31**, 29.
Bielschowsky, M. & Bielschowsky, F. (1956). *Aust. J. exp. Biol. med. Sci.* **34**, 181.
Brody, S. (1945). *Bioenergetics and Growth*, p. 352. New York: Reinhold Publishing Corp.
Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K. & Hastings, A. B. (1949). *J. biol. Chem.* **177**, 295.
Guggenheim, J. & Mayer, J. (1952). *J. biol. Chem.* **198**, 259.
Hughes, A. M. & Tolbert, B. M. (1958). *J. biol. Chem.* **231**, 339.
Ingalls, A. M., Dickie, M. M. & Snell, G. D. (1950). *J. Hered.* **41**, 317.
Mayer, J. (1953). *Physiol. Rev.* **33**, 472.
Mayer, J., Russell, R. E., Bates, M. W. & Dickie, M. M. (1952). *Endocrinology*, **50**, 318.
Sakami, W. (1955). *Handbook of Isotope Tracer Methods.* Ed. by Sakami, W. Cleveland, Ohio: Western Reserve University School of Medicine.
Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Shull, K. H., Ashmore, J. & Mayer, J. (1956). *Arch. Biochem. Biophys.* **62**, 210.
Shull, K. H. & Mayer, J. (1956). *J. biol. Chem.* **218**, 885.
Siegel, B. V. & Hughes, A. M. (1956). *Amer. J. Physiol.* **184**, 356.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.
Wertheimer, E. & Shapiro, B. (1948). *Physiol. Rev.* **28**, 451.

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A Protein Disulphide Reductase from Pea Seeds

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Sulphydryl groups play an important role in the reactions and properties of many proteins. The activity of many enzymes depends on the presence of sulphydryl groups; in addition to such functions as the binding of substrates or cofactors and participation as acyl acceptors, Swenson & Boyer (1957) and Boyer (1959) have suggested that sulphydryl groups may have a general role in maintaining proteins in a suitable configuration for enzyme activity. Oxidation of sulphydryl groups in proteins may explain oxygen-toxicity effects in whole tissues and extracts (Gerschman, Gilbert, Nye, Dyer & Fenn, 1954; Haugaard, Hess & Itskovitz, 1957; Turner & Mapson, 1958; Turner, Turner, Shortman & King, 1958; Hatch & Turner, 1959, 1960) and may be responsible in part for damage by ionizing radiations (Barron & Johnson, 1954; Ginzburg, Pandre & Binus, 1957; Hope, 1958; Clubb & Wills, 1959). There is evidence suggesting that the formation of protein-sulphydryl groups may precede cell division (Mazia, 1954; Nickerson & Falcone, 1956*b*; Kawamura & Dan, 1958; Sakai & Dan, 1959).

Nickerson & Falcone (1956*a*) reported that a particulate fraction from yeast reduced disulphide groups in a yeast cell-wall protein in the presence of a liver-coenzyme concentrate. Succinate and ethanol acted as hydrogen donors but the exact

nature of the reduction was not determined. During studies on the mechanism of the reversible aerobic inhibition of glycolysis in pea-seed extracts (Hatch & Turner, 1960) evidence was obtained for the reduction of protein-disulphide groups (especially those of glyceraldehyde 3-phosphate dehydrogenase) in the presence of reduced di- or tri-phosphopyridine nucleotide. A heat-labile component of the pea-seed extract was necessary for this reduction and the partial purification of this enzyme, protein disulphide reductase, and its properties are described in this paper.

EXPERIMENTAL

Preparation of protein disulphide reductase from pea seeds

Defatted pea-seed powder was prepared as described by Turner (1957), except that ether at 0° was used. The powder (60 g.) was extracted for 15 hr. with 200 ml. of 0·04 M-$NaHCO_3$ and 5 ml. of toluene. This and subsequent operations were carried out below 5°. The final pH of the extract was approx. 7·2. The extract was centrifuged at 1000 *g* for 10 min. and the supernatant further centrifuged at 20 000 *g* for 45 min. This preparation will be referred to as the crude supernatant. In the determination of protein disulphide (S.S) reductase activity in the crude supernatant, the protein was precipitated by treatment with saturated $(NH_4)_2SO_4$, pH 7·0, to 90% saturation, centrifuged at 20 000 *g*, dissolved in 0·01 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, and dialysed with stirring against 400 vol. of the same buffer. Cellulose dialysis tubing (Visking Corp., Chicago, Ill., U.S.A.) was used in all preparations.

The protein S.S reductase was partially purified by treating the crude supernatant with neutralized, saturated $(NH_4)_2SO_4$. The fraction obtained between 43 and 50% saturation was dissolved in 30 ml. of 0·01 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0. Acetone was added and the fraction precipitating between 0·85 and 1·4 vol. of acetone was separated by centrifuging at 5000 *g* for 20 min. During the acetone treatment the temperature was kept below −5°. The acetone fraction was taken up in 10 ml. of 0·01 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, and dialysed against 400 vol. of the same buffer for 3 hr.

Preparation of protein disulphide reductase from other plant tissues

Seeds of broad bean, pea, barley, maize and wheat were germinated and grown for 5–7 days in damp sand in a glass-house. The roots or shoots were excised and approx. 50 g. fresh wt. of the tissue was blended with 50 ml. of 0·05 M-$NaHCO_3$ and centrifuged at 20 000 *g* for 20 min. The supernatants were treated with saturated $(NH_4)_2SO_4$, pH 7·0, and the fraction precipitating between 38 and 55% saturation was dissolved in 0·01 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, and dialysed against the same buffer.

Soaked seeds of broad bean, wheat, barley, maize and oat, the laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) and potato tubers were also blended with 0·05 M-$NaHCO_3$ and treated as described above.

Materials

The following reagents were obtained from commercial sources: diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), hexokinase preparation (Pabst Laboratories, Milwaukee, Wis., U.S.A.), reduced diphosphopyridine nucleotide (DPNH), oxidized glutathione (GSSG), crystalline preparations of muscle glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, yeast alcohol dehydrogenase (C. F. Boehringer und Söhne, Mannheim, Germany), glucose 6-phosphate (G 6-P), *p*-chloromercuribenzoate (Sigma Chemical Co., St Louis, Mo., U.S.A.), cystine (L. Light and Co. Ltd.), cysteine (British Drug Houses Ltd.) and crystalline preparations of bovine-serum albumin, β-lactoglobulin, haemoglobin and ovalbumin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). Reduced triphosphopyridine nucleotide (TPNH) was prepared from TPN by the method of Hogeboom & Barry (1948), and was standardized spectrophotometrically at 340 mμ. Pea-seed glyceraldehyde 3-phosphate dehydrogenase was prepared as described by Hageman & Arnon (1955).

Preparation of pea-protein fraction for substrate

The pea-seed-protein fraction used as substrate for the assay of protein S.S reductase was prepared as follows. Defatted pea powder was extracted with a solution of $NaHCO_3$ as described for the preparation of protein S.S reductase. The supernatant after centrifuging at 20 000 *g* was treated with saturated $(NH_4)_2SO_4$, pH 7·0, and the fraction precipitated between 60 and 80% saturation was dissolved in water and dialysed against 200 vol. of mM-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, for 4 hr. at 5°. The extract was incubated in O_2 for 5 hr. at 20° to oxidize the remaining protein sulphydryl (SH) groups susceptible to aerobic oxidation. The preparation was free from protein S.S-reductase activity.

Analytical methods

Estimation of protein sulphydryl groups. The general procedure of Boyer (1954) was used for determination of protein SH groups. The increase in extinction at 255 mμ resulting from the reaction of SH groups with *p*-chloromercuribenzoate was determined in the presence of 0·05 M-KH_2PO_4–Na_2HPO_4 buffer, pH 6·3, and 0·25 M-Na_2SO_4. With concentrations of nucleotides greater than 15 μM the normal increase in extinction resulting from the reaction of protein SH groups and *p*-chloromercuribenzoate was not obtained, so that protein SH-group formation in the presence of substrate levels of DPNH or TPNH was determined by amperometric titration with $AgNO_3$ (Weissman, Schoenbach & Armistead, 1950).

Cystine reductase, glutathione reductase and glucose 6-phosphate dehydrogenase activity. Cystine reductase and GSSG reductase were assayed as described by Romano & Nickerson (1954) and Racker (1955) respectively. G 6-P-dehydrogenase activity was determined by measuring TPNH formation spectrophotometrically at 340 mμ in a reaction mixture containing: G 6-P, 30 μmoles; TPN, 0·9 μmole; KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, 45 μmoles; total vol. 3 ml.

Determination of protein. The protein content of enzyme and substrate preparations from pea seeds was estimated by the method of Warburg & Christian (1941).

Protein disulphide-reductase activity. Enzyme studies were carried out at 30° in Thunberg tubes gassed with O_2-free N_2. Protein S.S-reductase activity was determined from the increase in protein SH-group concentration in reaction mixtures of the following composition; enzyme and substrate as indicated in individual experiments; KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, 15 μmoles; G 6-P, 10 μmoles; G 6-P dehydrogenase, 18 μg.; TPN, 0·1 μmole; total vol., 1·1 ml. Modifications of this general procedure are indicated in the relevant tables or figures. The rate of reaction was linear until approx. 35 μequiv. of SH groups/g. of substrate protein were formed. For the assay of protein S.S-reductase activity, the reaction was allowed to proceed until approx. 10–15 μequiv. of SH groups/g. of substrate protein were produced.

RESULTS

Purification of protein disulphide reductase. Fractionation of a pea-seed extract with ammonium sulphate and acetone (see Experimental section) resulted in a more than 40-fold purification of protein S.S reductase. The results of a typical purification are shown in Table 1. Removal of protein, which was precipitated during storage of the acetone fraction at −15°, often increased the specific activity of the partially purified preparation to more than 60 times that of unfractionated extracts. Except where otherwise indicated the partially purified enzyme was used in subsequent studies.

Reduction of disulphide groups of a pea-protein fraction. In the presence of the partially purified enzyme and TPNH (generated by G 6-P dehydrogenase), S.S groups of the pea-seed-protein extract were reduced (Fig. 1). The rate fell to zero when the amount of protein-SH group increased by approx. 48 μequiv./g. of substrate protein. For more than 65 % of the observed SH-group formation the rate of reaction was approximately linear. Whether the reaction proceeded to completion was not determined as the initial concentration of protein S.S groups and their susceptibility to reduction were not known.

Table 1. *Purification of protein disulphide reductase*

The reaction mixtures were of the composition described for the assay of protein S.S reductase and contained 8 mg. of substrate protein. The amount of enzyme preparation added was adjusted to give not more than 10–15 μequiv. of SH groups/g. of substrate protein/hr. Temperature 30°.

Fraction precipitated by	Total units*	Protein concn. (mg./ml.)	Specific activity (units/mg. of protein)
0–90 % $(NH_4)_2SO_4$	95	35	0·09
43–50 % $(NH_4)_2SO_4$	83	7·3	0·99
0·85–1·4 vol. of acetone	43	2·3	3·7

* A unit of protein disulphide reductase was defined as the amount of enzyme which catalysed the formation of 0·1 μequiv. of SH group/hr.

Requirement for reduced di- and tri-phosphopyridine nucleotide. In the presence of systems which produced either DPNH or TPNH, protein S.S groups of unfractionated pea-seed extracts were reduced (Hatch & Turner, 1960). The partially purified protein S.S reductase also utilized both DPNH and TPNH (Table 2). It was reported previously that pyridine nucleotide transhydrogenase was not detected in the pea-seed extracts (Hatch & Turner, 1960). With TPNH as hydrogen donor the rate of protein S.S reduction was approximately twice that with DPNH. A similar ratio was

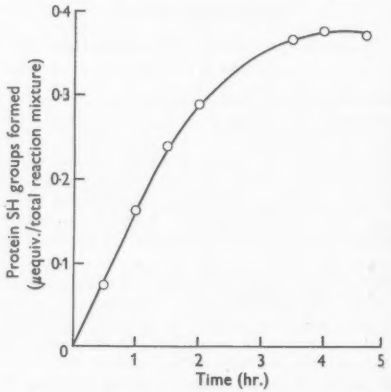

Fig. 1. Reduction of protein S.S groups by TPNH. The reaction mixtures (containing 0·38 mg. of enzyme protein and 8 mg. of substrate protein) were of the composition described for the assay of protein S.S reductase. Temperature 30°.

Table 2. *Requirement of reduced di- and tri-phosphopyridine nucleotides for protein disulphide-reductase activity*

The reaction mixtures of Expt. 1 contained 0·24 mg. of enzyme protein and 11 mg. of substrate protein and were of the composition described for the assay of protein S.S reductase. The reaction mixtures of Expt. 2 contained: enzyme protein, 1·3 mg.; substrate protein, 18 mg.; KH_2PO_4–Na_2HPO_4 buffer (pH 7·0), 25 μmoles; DPNH, 4·8 μmoles, or TPNH, 4·0 μmoles, added as shown; total vol. was 2·2 ml. Incubation time, 1 hr. Temperature 30°.

Expt. no.	Reaction-mixture composition	Protein SH groups formed (μequiv./reaction mixture)
1	Complete system	0·11
	No G 6-P dehydrogenase	0·12
	No G 6-P	0
	No TPN	0
2	Complete system with DPNH	0·18
	Complete system with TPNH	0·32
	No DPNH or TPNH	0

obtained with crude enzyme extracts, which suggested that the activity of crude and partially purified preparations was due to a single enzyme. Protein S.S groups were also reduced by TPNH generated by G 6-P dehydrogenase (Table 2). Although the partially purified protein S.S-reductase preparation generally contained sufficient G 6-P dehydrogenase, additional enzyme was added to ensure that TPNH supply was not rate-limiting. In separate experiments no oxidation of protein SH groups was observed in reaction mixtures containing enzyme, substrate protein not pretreated in oxygen and substrate amounts of DPN or TPN.

The K_m for TPNH was 2 μM. This value was calculated from the Lineweaver–Burk plot (Lineweaver & Burk, 1934) of the rates of protein S.S reduction in the presence of various concentrations of added TPN and an excess of G 6-P and G 6-P dehydrogenase (Fig. 2). It was assumed that the concentration of TPNH in the reaction mixtures approximated to the concentration of added TPN. A dehydrogenase with a suitable equilibrium was not available for determining the K_m for DPNH.

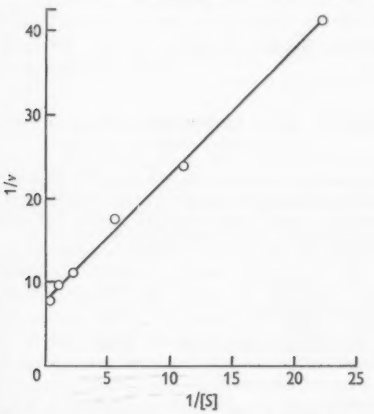

Fig. 2. Lineweaver–Burk plot of $1/v$ against $1/[S]$ for protein S.S reductase. v is the initial velocity (μequiv. of SH groups formed/reaction mixture/hr.); $[S]$ is $10^5 \times$ concn. (M) of TPNH. The reaction mixtures (containing 0·6 mg. of enzyme protein, 11 mg. of substrate protein and varying amounts of TPN) were of the composition described for the assay of protein S.S reductase. Incubation time, 1 hr. Temperature 30°.

Relationship of protein disulphide-reductase activity to cystine reductase and glutathione reductase

Previous observations suggested that neither cystine reductase nor GSSG reductase was responsible for the reduction of protein S.S groups by crude pea extracts (Hatch & Turner, 1960). This conclusion was supported by the observation that during the purification of protein S.S reductase, comparable purification of cystine reductase and GSSG reductase was not obtained (Table 3). Protein S.S reductase was purified more than 40-fold compared with a twofold purification of GSSG reductase. Cystine reductase was completely removed by the fractionation procedure.

Reduction of disulphide groups of other protein preparations. The enzyme showed some specificity towards different proteins, the rate of reduction of their S.S groups varying considerably (Table 4). Some proteins were not attacked whereas with others only a small fraction of the S.S groups in the protein preparations was reduced. The concentration of substrate protein may have influenced the activity observed.

Effect of pH. Protein S.S-reductase activity was optimum between pH 6·9 and 7·4 and declined rapidly on either side of this range (Fig. 3). Some precipitation of protein from the reaction mixtures occurred at pH 6·2.

Effect of inhibitors. The effect of several enzyme inhibitors on protein S.S-reductase activity was examined (Table 5). *p*-Chloromercuribenzoate, iodoacetate, Hg^{2+} ions, Ag^{+} ions and Cu^{2+} ions, which are known to affect SH groups, inhibited activity. The enzyme was also inactivated by

Table 3. *Effect of fractionation procedure for protein disulphide reductase on the activity of glutathione reductase and cystine reductase*

The various enzyme activities were assayed as described in the Experimental section. The reaction mixtures for the assay of protein S.S-reductase activity contained 8 mg. of substrate protein and the amount of enzyme fraction added was adjusted to give not more than 10–15 μequiv. of SH groups/g./hr. Temperature 30°.

Fraction precipitated by	Protein S.S-reductase activity (units/mg. of enzyme protein)*	GSSG-reductase activity (extinction change/mg. of enzyme/min.)	Cystine-reductase activity (extinction change/mg. of enzyme/min.)
0–90% $(NH_4)_2SO_4$	0·065	0·025	0·003
43–50% $(NH_4)_2SO_4$	0·70	0·184	0
0·85–1·4 vol. of acetone	2·8	0·053	0

* Units as defined in Table 1.

Table 4. *Reduction of disulphide groups of various protein preparations*

The reaction mixtures were of the composition described for the assay of protein S.S reductase. Expts. 1–3 contained 0·38, 0·40 and 0·34 mg. of enzyme protein respectively; amounts of substrate protein are shown in the table. The substrate-protein solutions were incubated in O_2 for 5 hr. at 20° to oxidize SH groups. Reaction time, 1 hr.

Expt. no.	Substrate protein	Amount (mg./total reaction mixture)	Protein SH groups formed (μequiv./reaction mixture)*
1	Cryst. glyceraldehyde 3-phosphate dehydrogenase	5	0·025
	Cryst. alcohol dehydrogenase	3	0·086
	Cryst. aldolase	2·5	0
	Hexokinase preparation	4	0·038
2	Cryst. ovalbumin	3·5	0
	Cryst. serum albumin	10	0·024
	Cryst. haemoglobin	6	0
	β-Lactoglobulin	6	0
3	Pea-seed glyceraldehyde 3-phosphate-dehydrogenase preparation	12	0·092

* No activity was found in the absence of the enzyme preparation.

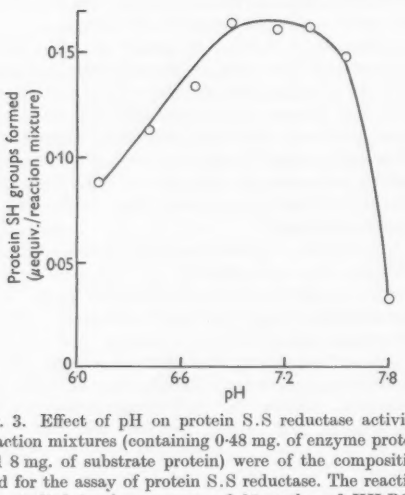

Fig. 3. Effect of pH on protein S.S reductase activity. Reaction mixtures (containing 0·48 mg. of enzyme protein and 8 mg. of substrate protein) were of the composition used for the assay of protein S.S reductase. The reaction was studied in the presence of 15 μmoles of KH_2PO_4–Na_2HPO_4 buffer of the pH indicated. Incubation time was 1 hr. and the temperature was 30°.

Table 5. *Effect of enzyme inhibitors on protein disulphide-reductase activity*

The reaction mixtures, which contained 0·30–0·40 mg. of enzyme protein and 9 mg. of substrate protein, were of the composition used for the assay of protein S.S reductase. Incubation time, 1 hr. Temperature 30°.

Inhibitor	Concn. (mM)	Inhibition (%)
Sodium molybdate	0·1	0
Sodium cyanide	10	0
Ethylenediaminetetra-acetate	0·045	0
Sodium fluoride	10	0
p-Chloromercuribenzoate	0·054	88
Iodoacetate	0·054	57
Sodium arsenite	0·15	100
Sodium arsenite	0·045	89
Cadmium sulphate	0·005	91
Zinc sulphate	0·003	82
Mercuric chloride	0·002	92
Silver nitrate	0·009	66
Cupric sulphate	0·015	100

arsenite and Cd^{2+} ions; at low concentrations these reagents are considered to be specific for reactions involving a dithiol grouping (Sanadi, Langley & White, 1959). Zn^{2+} ions (3 μM) strongly inhibited the enzyme. At the concentrations used in these experiments the SH and dithiol reagents did not interfere with the estimation of protein SH groups. When G 6-P-dehydrogenase activity was examined under the same conditions as those used for the assay of protein S.S reductase, Ag^+ ions, Hg^{2+} ions and *p*-chloromercuribenzoate inhibited the enzyme by 37, 48 and 15% respectively; Zn^{2+} ions, Cu^{2+} ions, Cd^{2+} ions, arsenite and iodoacetate did not affect the activity. The results obtained for protein S.S-reductase inhibition were therefore not due to the inhibition of G 6-P dehydrogenase as reaction mixtures for the assay of protein S.S reductase contained excess of this enzyme.

Enzyme stability. Protein S.S-reductase preparations could be stored at −15° for at least 8 weeks without loss of activity. Heating at 60° for 10 min. had no effect, but at 70° the enzyme was completely inactivated. Adjustment of the pH to 5·5 for 5 min. at room temperature or at 5° irreversibly inactivated the enzyme.

Distribution in plant tissues

Protein S.S-reductase activity was detected in extracts from the following tissues: broad bean, wheat, barley, maize and oat seeds; shoots of pea, broad bean, maize, wheat and barley seedlings and roots of pea, barley and maize seedlings. With extracts from barley and oat seeds, and pea, wheat and barley shoots, activity was increased by the addition of 0·035 mM-ethylenediaminetetra-acetate (EDTA). In these extracts heavy-metal ions probably affected activity. However, extracts from broad-bean roots, silver-beet leaves and potato tubers were inactive in the presence or absence of EDTA. In all cases activity was completely inhibited by 0·2 mM-sodium arsenite, indicating that neither cystine reductase nor GSSG reductase was involved. In separate experiments it was found that pea GSSG reductase was only slightly inhibited by 0·5 mM-arsenite whereas cystine reductase was not affected.

DISCUSSION

The present investigation has shown that active preparations of an enzyme catalysing the reduction of protein S.S groups by reduced pyridine nucleotides may be readily obtained from pea seeds. This enzyme, protein disulphide reductase, acted on the S.S groups of several enzyme and other protein preparations and may thus be of considerable importance in cell metabolism in reducing the S.S groups of these (and probably other) proteins.

The purification data and the effect of arsenite as an inhibitor indicated that neither cystine reductase (Romano & Nickerson, 1954) nor GSSG reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951) was involved in the reduction of protein S.S groups. Protein S.S reductase was irreversibly inactivated at pH 5·5, whereas Moustafa (1955) found that pea GSSG reductase was stable at this pH. Boyer (1959) suggested that GSSG reductase or cystine reductase could reduce protein S.S groups in the presence of catalytic amounts of substrate. Pihl, Eldjarn & Bremer (1957) found that liver GSSG reductase catalysed the reduction of a number of non-protein S.S compounds if a catalytic amount of GSH was added, electrons being transferred non-enzymically from GSH to the S.S compounds.

It was not possible to determine the equilibrium constant for the reaction between reduced pyridine nucleotides and protein S.S groups. No oxidation of protein SH groups was observed in reaction mixtures containing substrate levels of DPN or TPN and from this it appeared that the reaction equilibrium for protein S.S reductase favours the reduction of protein S.S groups. This conclusion is supported by the experiments of Rall & Lehninger (1952), who found that GSSG was almost completely reduced by TPNH in the presence of GSSG reductase. Boyer (1959) has pointed out that at least some of the SH–S.S groups in proteins have electrode potentials close to those for low-molecular-weight thiols such as GSH. The values of the electrode potentials of SH–S.S systems (and therefore the expected degree of reduction of S.S groups by TPNH) are in doubt (Calvin, 1954; Boyer, 1959).

Inhibition of protein S.S reductase by SH-group reagents and by low concentrations of the dithiol reagents, arsenite and Cd^{2+} ions, indicated that SH groups have a primary role in the activity of the enzyme. Arsenite and Cd^{2+} ions are specific inhibitors of reactions involving the dithiol grouping (Sanadi *et al.* 1959). A number of enzymes are inhibited by reagents which combine with monothiols but are not affected by low concentrations of arsenite (James, 1953). Arsenite forms only a weak complex with monothiols but a stable ring structure with dithiol compounds, provided that the SH groups are spatially adjacent (Stocken & Thompson, 1946; James, 1953). Inhibition of protein S.S reductase by arsenite and Cd^{2+} ions suggests that the enzyme contains spatially adjacent SH groups at the catalytically active site. Although it might be anticipated that protein SH groups in this configuration would be susceptible to oxidation to the S.S form in the presence of molecular oxygen (Boyer, 1959), the enzyme remained active even after prolonged pretreatment in air or oxygen. A mechanism of action for the enzyme involving the alternate oxidation and reduction of the catalytically active dithiol grouping would be consistent with this observation and with the inhibition by arsenite and Cd^{2+} ions. If such a mechanism operates, the steps leading to the reduction of substrate–protein S.S groups may involve: (*a*) reduction of the S.S groups at the enzyme surface to a dithiol by DPNH or TPNH, (*b*) the transfer of electrons from the dithiol group to the S.S group of the substrate protein, leaving the enzyme in the S.S form.

Liver GSSG reductase and the pea-protein S.S reductase have some properties in common. Langdon (1958) showed that GSSG reductase was inhibited by low concentrations of Zn^{2+} ions and *p*-chloromercuribenzoate. Inhibition by Zn^{2+} ions was observed only if the enzyme was pretreated with the reagent, and TPNH and GSSG protected the enzyme. The results suggested that Zn^{2+} ions were chelated at the catalytically active site by groups responsible for the binding of GSSG and TPNH to the enzyme. Langdon proposed a two-step reduction of GSSG by TPNH. The scheme involved the transfer of an electron from an enzyme SH group to give GSH and an enzyme–SSG

complex which was then reduced by TPNH to give enzyme-SH plus GSH. With protein S.S reductase, TPNH and substrate protein did not prevent inhibition by Zn^{2+} ions, which suggested that the enzymes operated by different mechanisms.

SUMMARY

1. Protein disulphide reductase, which catalyses the reduction of protein disulphide groups, was purified approximately 40-fold from pea-seed extracts.

2. The enzyme utilized reduced di- and triphosphopyridine nucleotide to reduce protein-disulphide groups. The rate of reaction with reduced triphosphopyridine nucleotide was approximately twice that with reduced diphosphopyridine nucleotide.

3. A protein fraction from pea seeds was used as substrate in most experiments. The enzyme also reduced disulphide groups of a pea-seed glyceraldehyde 3-phosphate-dehydrogenase preparation and of some other enzymes and proteins.

4. Cystine reductase and glutathione reductase, both of which were present in crude pea-seed extracts, were not involved in the reduction of protein-disulphide groups.

5. The effects of pH and concentration of reduced triphosphopyridine nucleotide on the enzyme activity were studied.

6. Protein disulphide-reductase activity was strongly inhibited by low concentrations of *p*-chloromercuribenzoate, iodoacetate, Hg^{2+} ions, Ag^{+} ions, Cu^{2+} ions, Cd^{2+} ions, Zn^{2+} ions and arsenite.

7. Active preparations of the enzyme were obtained from a number of plant tissues.

8. A possible mechanism of action of protein disulphide reductase is presented.

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation and Transport, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Dr R. N. Robertson for his interest during the course of the investigation and to Dr J. R. Vickery, Chief, Division of Food Preservation and Transport, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

REFERENCES

Barron, E. S. G. & Johnson, P. (1954). *Arch. Biochem. Biophys.* **48**, 149.

Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.

Boyer, P. D. (1959). In *The Enzymes*, 2nd ed., p. 514. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.

Calvin, M. (1954). In *Glutathione*, p. 3. Ed. by Colowick, S., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H. New York: Academic Press Inc.

Clubb, M. E. & Wills, E. D. (1959). *Biochem. J.* **71**, 16P.

Conn, E. E. & Vennesland, B. (1951). *J. biol. Chem.* **192**, 17.

Gerschman, R., Gilbert, D. L., Nye, S. W., Dyer, P. & Fenn, W. O. (1954). *Science*, **119**, 623.

Ginzburg, M. B., Pandre, E. M. & Binus, N. M. (1957). *Biokhimiya*, **22**, 467.

Hageman, R. H. & Arnon, D. I. (1955). *Arch. Biochem. Biophys.* **55**, 162.

Hatch, M. D. & Turner, J. F. (1959). *Biochem. J.* **72**, 524.

Hatch, M. D. & Turner, J. F. (1960). *Biochem. J.* **75**, 66.

Haugaard, N., Hess, M. E. & Itskovitz, H. (1957). *J. biol. Chem.* **227**, 605.

Hogeboom, G. H. & Barry, G. T. (1948). *J. biol. Chem.* **176**, 935.

Hope, D. B. (1958). *Biochem. J.* **68**, 37P.

James, W. O. (1953). *Annu. Rev. Pl. Physiol.* **4**, 59.

Kawamura, N. & Dan, K. (1958). *J. biophys. biochem. Cytol.* **4**, 615.

Langdon, R. G. (1958). *Biochim. biophys. Acta*, **30**, 432.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Mapson, L. W. & Goddard, D. R. (1951). *Biochem. J.* **49**, 592.

Mazia, D. (1954). In *Glutathione*, p. 209. Ed. by Colowick, S., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H. New York: Academic Press Inc.

Moustafa, E. M. (1955). *Proc. Egypt. Acad. Sci.* **11**, 20.

Nickerson, W. J. & Falcone, G. (1956*a*). *Science*, **124**, 318.

Nickerson, W. J. & Falcone, G. (1956*b*). *Science*, **124**, 722.

Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.

Racker, E. (1955). *J. biol. Chem.* **217**, 855.

Rall, T. W. & Lehninger, A. L. (1952). *J. biol. Chem.* **194**, 119.

Romano, A. H. & Nickerson, W. J. (1954). *J. biol. Chem.* **208**, 409.

Sakai, H. & Dan, K. (1959). *Exp. Cell Res.* **16**, 24.

Sanadi, D. R., Langley, M. & White, F. (1959). *J. biol. Chem.* **234**, 183.

Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 529.

Swenson, A. D. & Boyer, P. D. (1957). *J. Amer. chem. Soc.* **79**, 2174.

Turner, J. F. (1957). *Biochem. J.* **67**, 450.

Turner, J. F. & Mapson, L. W. (1958). *Nature, Lond.*, **181**, 270.

Turner, J. S., Turner, J. F., Shortman, K. D. & King, J. E. (1958). *Aust. J. biol. Sci.* **11**, 336.

Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

Weisman, N., Schoenbach, E. G. & Armistead, E. B. (1950). *J. biol. Chem.* **187**, 153.

Studies on Vitamin E

4. THE SIMULTANEOUS DETERMINATIONS OF TOCOPHEROLS, UBIQUINONES AND UBICHROMENOLS (SUBSTANCE SC) IN ANIMAL TISSUES: A RECONSIDERATION OF THE KEILIN–HARTREE HEART PREPARATION*

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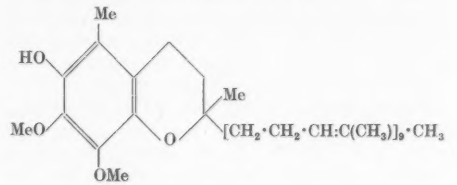
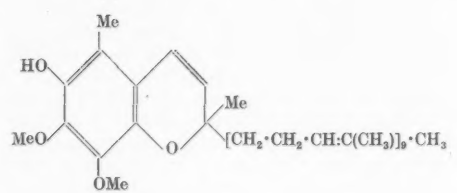
(*Received* 5 *February* 1960)

Bouman & Slater (1957), in a study of the Keilin–Hartree heart preparation, observed that it contained appreciable amounts of a substance they believed to be α-tocopherol. They found further that by treating the lipid extract with a mixture of ascorbic acid and ethanolic hydrochloric acid, a naturally occurring quinone, thought to be tocopherylquinone, was reduced and cyclized to a new substance apparently identical with α-tocopherol (criteria being ultraviolet spectrum and chromatography on zinc carbonate-treated paper). More recently, Bouman, Slater, Rudney & Links (1958) have reconsidered the interpretation of their original observations, in view of their findings that ubiquinone 50, a naturally occurring quinone [in particularly high concentration in normal heart, see Lester & Crane (1959)] could be reduced and cyclized to give a product with the same ultraviolet maximum and apparently the same chromatographic behaviour as α-tocopherol. The nature of this product has not been confirmed, but it may have the structure of the chromanol (I). In a personal communication, Professor R. A. Morton has informed us that the nature of the cyclized products may depend on the reagents used, certain reagents apparently causing alterations in the side chain. We have therefore preferred for the present to call the product RC-ubiquinone. Bouman *et al.* (1958) concluded that the formation of this product accounted quantitatively for the earlier results of Bouman & Slater (1957) and were of the opinion that the presence of α-tocopherylquinone in heart remained to be established.

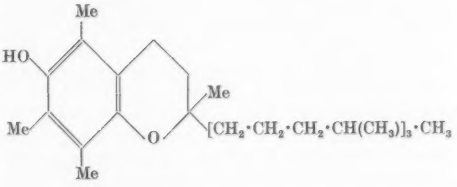
Several factors combined to urge a reinvestigation of these findings. Our own studies indicated that the chromatographic data given by Bouman *et al.* (1958) could hardly be correct. Work by Edwin, Diplock, Bunyan & Green (1960) has shown that animal tissues contain a reducing substance that simulates α-tocopherol (from which it can, however, be separated by two-dimensional paper chromatography) and that this substance runs near ubiquinone on chromatograms. Meanwhile Laidman, Morton, Paterson & Pennock (1959) have shown that a reducing substance isolated from human kidney and from several other animal tissues (Lowe, Morton & Harrison, 1953; Cunningham & Morton, 1959) has the structure (II).



(I)



(II)



(III)

This substance, previously designated by Morton and co-workers as substance SC, and now called by them ubichromenol, is obviously related to RC-ubiquinone 50 and may indeed be the 3:4-dehydro derivative of the latter. Its chemical similarities to α-tocopherol (III) are equally apparent and the experiments that follow indicate that the ubichromenols are the main non-tocopherol-reducing substances remaining in animal tissues after classical methods of purification of tissue extracts for vitamin E analysis. This paper compares the

* Part 3: Bunyan, Green, Edwin & Diplock (1960).

chromatographic behaviour of ubiquinones, ubichromenols, RC-ubiquinone, tocopherols and certain other substances. It also describes an analytical procedure for the simultaneous determination of the three groups of compounds. The Keilin–Hartree heart preparation has been reinvestigated.

EXPERIMENTAL AND RESULTS

Nomenclature

The term ubiquinone was originally proposed by Morton, Wilson, Lowe & Leat (1957) to describe a quinone isolated from liver. It is now known (Lester, Crane & Hatefi, 1958) that a series of such quinones exist in Nature, and five compounds, differing from each other only by isoprenoid units, have already been isolated. The normal ubiquinone found in higher mammalian tissues, such as ox and pig heart, has been considered by Lester & Crane (1959) to have a C_{50} side chain (see also Morton *et al.* 1958), but recently Rüegg *et al.* (1959) have shown that the ubiquinone of rat liver is the C_{45} 'isoprenologue', and Diplock, Edwin, Green, Bunyan & Marcinkiewicz (1960) have demonstrated that the C_{50}, C_{40} and C_{35} compounds also exist in rat liver. In the text below we have indicated, wherever possible, which ubiquinone is being examined by giving the number of carbon atoms in the side chain; thus the compound from pig heart is ubiquinone 50. Where the term ubiquinone is used without a number, either its identity is obvious from the text or else it is used in a generic sense and applies to any of the group of compounds. The term ubichromenol refers to the cyclized chromenol, formally derived from the ubiquinone which is isomeric with it; thus ubichromenol 50 contains a C_{47} side chain etc. Diplock *et al.* (1960) have found that a series of such ubichromenols also exists in animal tissues, the chief one in rat liver being ubichromenol 45. Lester *et al.* (1958) have called the series of ubiquinones, coenzymes Q, but one advantage of the ubiquinone terminology is the convenience with which the quinones may be related to the isomeric ubichromenols.

Materials

Ubiquinone 50 and ubiquinone 30 were kindly given by Professor R. A. Morton.

Ubichromenol 45. This was prepared from the livers of vitamin E-deficient rats, which were a convenient source since an otherwise difficult separation from tocopherol could be avoided. The livers (400 g.) from rats were homogenized at $-70°$ by the sodium sulphate–acetone method of Edwin *et al.* (1960). The lipid was extracted with acetone in a Soxhlet apparatus for 3 hr. and then saponified, sterols were removed and the material was purified by the methods described for vitamin E analysis (Edwin *et al.* 1960). The total extract was chromatographed on 60 cm.-wide $ZnCO_3$-treated paper, after pilot strips had shown that the concentration was low enough to prevent co-chromatography of the ubichromenol with ubiquinone (see below). After a 1 hr. run in *cyclo*hexane–benzene, the bands were viewed under u.v. light and the ubichromenol band was removed. Elution from the paper with ethanol gave ubichromenol ($\lambda_{max.}$ 275 mμ) still somewhat contaminated with ubiquinone. It was further purified by chromatography on a 20 cm. × 1·5 cm. column of alumina [Peter Spence and Sons Ltd., type O; see Cunningham & Morton (1959)]. The ubiquinone was eluted with 10% (v/v) of ethanol in ether. The ubichromenol remained as a pink band on the column and was eluted with 15% (v/v) of ethanol in ether. The material was freed from traces of ubiquinone by two more separations on alumina. The final eluate, on evaporation in N_2, gave ubichromenol as a pale-yellow oil (1·4 mg.). Its u.v. spectrum was very similar to that given for the pure substance by Cunningham & Morton, with $\lambda_{max.}$ at 233, 275 and 332 mμ, an inflexion at 281 mμ and $\lambda_{min.}$ at 259 and 302 mμ. $E^{1\%}_{1\,cm.}$ for the preparation was 71, whereas Professor Morton has informed us that $E^{1\%}_{1\,cm.}$ for pure ubichromenol 50 is 96·1.

Chromatographic studies on zinc carbonate-treated paper

RC-*ubiquinone*. Bouman & Slater (1957) have recorded almost identical R values for the migration of α-tocopherol, ubiquinone 50, RC-ubiquinone 50 and ubiquinol 50 on $ZnCO_3$-treated paper (0·71, 0·77, 0·73 and 0·73 respectively). [Although several workers have preferred to use the term 'R_F value', as defined by Consden, Gordon & Martin (1944), for paper-partition chromatography, we have found it convenient to use the term 'R value' to describe migrations on $ZnCO_3$-treated paper. This is in accordance with the original terminology Le Rosen (1942) applied to adsorption columns where, as on $ZnCO_3$-treated papers, migrations are conventionally measured from the position of maximum concentration, i.e. the head of the band. Moreover, there is some convenience in being able to distinguish in this way between adsorption and partition runs.] It seemed likely, however, that these figures were in part incorrect, if the likely effect of the structural groups that are substituents in the ring system of RC-ubiquinone are considered. In RC-ubiquinone, the accelerating effect of the long unsaturated side chain (compared with α-tocopherol) would be expected to be overcome by a pronounced retarding effect of the nuclear methoxy groups. Thus on $ZnCO_3$-treated paper, with benzene as solvent, the R values of *o*-, *m*- and *p*-cresol are 0·63, 0·44 and 0·44 respectively and those for 2-, 3- and 4-methoxyphenol are 0·26, 0·33 and 0·33 respectively. The *o*-methoxy group, it should be noted, unlike the *o*-methyl group, retards chromatographic migration, probably owing to complexing of the two vicinal oxygen atoms to the zinc atoms through a bridge structure. It would thus be expected that RC-ubiquinone would have an R value in adsorption chromatography nearer to that of γ-tocopherol than to α-tocopherol. When RC-ubiquinone 50 was prepared by the ascorbic acid–HCl method of Bouman & Slater (1957), the product contained only about 20% of the desired substance, the remainder being unchanged ubiquinone. When this product was chromatographed in amounts not exceeding 10 μg., the RC-ubiquinone separ-

ated and migrated to a position near that of γ-tocopherol (R 0·45). When large amounts were run, RC-ubiquinone co-chromatographed with ubiquinone and had R 0·70, thus explaining the findings of Bouman & Slater.

Ubiquinones and ubichromenol. Both ubiquinone 50 and ubiquinone 30 have R values on $ZnCO_3$-treated paper of about 0·75 (slightly faster than α-tocopherol). When ubiquinone is chromatographed together with small quantities of tocopherols, RC-ubiquinone or ubichromenol, all these latter substances may co-chromatograph with the ubiquinone, thus exhibiting spurious R values. Ubichromenol is chromatographically indistinguishable from the reduced and cyclized compound prepared from the ubiquinone of the same molecular weight, in the systems used by us.

Separation of ubiquinones, ubichromenols, tocopherols and some other substances by two-dimensional paper chromatography

When preparations of animal tissue (purified by the methods described below) containing ubiquinones, ubichromenols, tocopherols, vitamin K_1 and α-tocopherylquinone are chromatographed in the two-dimensional adsorption–partition system of Green, Marcinkiewicz & Watt (1955), with 95% ethanol as mobile phase in the second dimension, the substances separate as shown schematically in Fig. 1. Provided that the preliminary steps of purification are rigorously carried out (in particular no traces of fat must remain), the factor that determines the separation pattern is the amount of the main ubiquinone present. As shown by Diplock *et al.* (1960) there may be more than one ubiquinone present in the tissue (particularly so in liver), but there is always one in preponderance. The amount of this ubiquinone is nearly always much greater than that of the other lipid constituents in the extract and because of its high molecular weight, it materially affects the partition not only of other substances but also of itself, this in addition to the co-chromatography sometimes observed in the first dimension. Thus ubichromenols, α-tocopherylquinone and β- and γ-tocopherol, whose true positions are shown by the full lines in Fig. 1, may occasionally occupy the false (but easily recognizable) positions shown by the dotted lines. This in no way interfered with the subsequent removal and determination of the substances, but should better separation be required, it is often sufficient to run the chromatogram again at a lower loading.

The only substantial modification to the chromatographic separation that may be necessary is when an extract from a tissue such as liver is being examined and there is reason to believe that small quantities of lower-molecular-weight ubiquinones or ubichromenols may be present. As indicated above, these mixtures are usually only partially separable on the normal chromatograms. Complete separation can be obtained, however, if the partition run is extended to about 16 hr. and the second dimension of the chromatographic paper increased to about 35 cm. Fig. 2 shows schematically the separation of a rat-liver extract into four ubiquinones and three ubichromenols, after separation on the normal square sheet of paper had shown only one of each type of compound. Under these long-running conditions, provided that the loading is light enough, the molecular weight of each compound can be found by the addition of known markers or by calculation from R_M values.

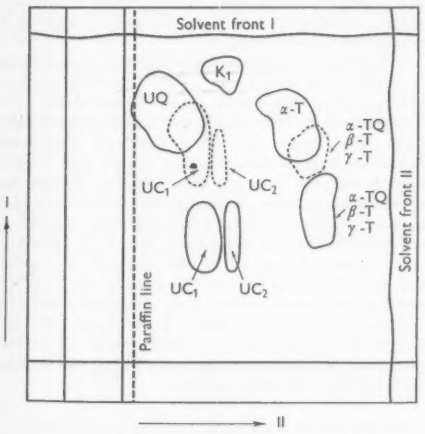


Fig. 1. Two-dimensional paper chromatography of preparations of animal tissues. The positions illustrated by the broken-line spots are those normally occupied by the substances indicated if much ubiquinone is present in the extract (for explanation, see text). K_1, Vitamin K_1; UQ, ubiquinone; UC_1 and UC_2, ubichromenols; α-T, α-tocopherol; α-TQ, α-tocopherylquinone; β-T, β-tocopherol; γ-T, γ-tocopherol.

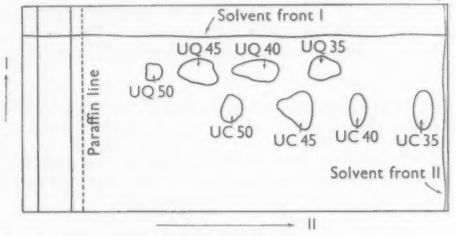


Fig. 2. Separation of a rat-liver extract by extending the partition run and increasing the second dimension of the chromatographic paper. UQ 50, 45, 40, 35, ubiquinones 50, 45, 40, 35 respectively. UC 50, 45, 40, 35, ubichromenols 50, 45, 40, 35 respectively.

All the ubiquinones, ubichromenols, tocopherols and other quinones are readily observed on the dried chromatograms under ultraviolet light. On paper treated with sodium fluorescein (Green, 1959) the limiting amounts observable are: tocopherol and all the quinones, about 1–2 μg; ubichromenols, 5–10 μg.

Colorimetric determination of ubichromenols

Ubichromenols can be determined by the ferric chloride–dipyridyl reaction in the same way as α-tocopherol. Under the conditions of oxidation normally used in these laboratories (Report of the Vitamin E Panel, S.A.C., 1959), the reaction is complete in 2 min., and extinctions at 520 mμ are linear with respect to concentration over the range usually encountered. The spectroscopic factor for the reaction of ubichromenol 45 is 276 at 520 mμ, compared with 98 for α-tocopherol. In view of the molecular-weight ratio between the two substances, it would appear therefore that less Fe^{2+} ion is produced in the oxidation of ubichromenol than in that of α-tocopherol [but see Boyer (1951) for a discussion of the influence of conditions on the mechanism of the ferric chloride–dipyridyl oxidation]. Since Kaunitz & Beaver (1944) found that the ferric chloride oxidation of α-tocopherol is influenced by the presence of certain lipid substances, a study was made of the effect of relatively large amounts of ubiquinone on the determination of ubichromenol (Table 1). Only very slight effects on the colour reaction were observed.

Simultaneous determination of ubiquinones, ubichromenols, tocopherols and vitamin A in animal tissues

The method of analysis is based on that described by Edwin *et al.* (1960) for tocopherols, except that purification is carried out on columns of Decalso F by a modification of the procedure of Crane, Lester, Widmer & Hatefi (1959) and the final paper-chromatographic separation is slightly altered.

(i) The tissue (20 g.) was homogenized with Na_2SO_4–acetone at −70° and extracted in the dark with acetone for 3 hr. After evaporation of the solvent the lipid extract was saponified in the presence of pyrogallol. It is important that saponification is complete. This can be assured by still further increasing the concentration of alkali and extending the time. For each gram of lipid or less, the saponification mixture consisted of 4 ml. of 5% (w/v) pyrogallol in ethanol and 2 ml. of saturated aqueous KOH solution. The mixture was heated in a boiling-water bath for 5 min. and then extracted with ether as usual. One-tenth of the non-saponifiable extract may be removed at this stage for measurement of vitamin A by the $SbCl_3$ method.

(ii) The remainder was dissolved in methanol (10 ml.) and sterols were removed at −15°. Where necessary, and particularly if the sterols after centrifuging were tinged with yellow, the solids were redissolved in a further 1–3 ml. of methanol and recrystallized once or twice.

(iii) The methanol-soluble fraction, after evaporation of solvent, was dissolved in light petroleum (b.p. 40–60°, 5 ml.). This was added to a column of Decalso F (5 g. in a tube 1·3 cm. in diameter) previously washed with the same solvent. After percolation of the extract at a rate of 1–1·5 ml./min. (controlled by a capillary outlet to the tube) the column was washed with a further 25 ml. of light petroleum and then eluted with benzene (35 ml.). The benzene eluate was evaporated at reduced pressure and the residue was dissolved in benzene (1 ml.) for paper chromatography.

(iv) Two-dimensional paper chromatography was carried out on $\frac{1}{4}$–$\frac{1}{2}$ of the total extract, as described by Edwin *et al.* (1960), except that 95% (v/v) ethanol was used as mobile phase for the second dimension. After the running, the papers were dried in a current of air (usually about 1 min.) and the spots are observed under u.v. light. Another chromatogram was sprayed heavily with the ferric chloride–dipyridyl reagent (freshly prepared) to indicate the positions of tocopherol and ubichromenol. As far as possible, exposure of papers and ubiquinone solutions to daylight should be avoided.

(v) Each clearly separated spot was cut out and eluted. Tocopherols were eluted with 3·5 ml. of 0·07% (w/v) αα′-dipyridyl in ethanol for the usual determination. Ubichromenols, if distinct, were eluted separately and determined with ferric chloride. Combined ubiquinone-ubichromenol spots were normally eluted with 6 ml. of ethanol; half the solution was used for ubichromenol determination and the remainder diluted with ethanol (if necessary) and the ubiquinone determined on a portion by the borohydride-assay method of Crane *et al.* (1959). To 3 ml. of solution was added 0·03 ml. of the KBH_4 solution. These proportions, as indicated by Crane *et al.*, are critical if turbidity is to be avoided. $E^{1\%}_{1\,cm.}$ (oxidized–reduced) at 274·5 mμ was found to be 140 on a pure specimen of ubiquinone 50. Tocopherylquinone and vitamin K_1 are only rarely observed (see below), but when visible can be determined by their ultraviolet spectra.

Table 1. *Effect of ubiquinone* 50 *on the ferric chloride–dipyridyl determination of ubichromenol* 45

Each substance or mixture of substances was dissolved in ethanol and the determination was in a final volume of 4 ml. of ethanol in a cell of 1 cm. light path.

Ubichromenol (μg.)	Ubiquinone added (μg.)	$E_{520\ m\mu}$
75·4	—	0·208
75·4	230	0·192
75·4	1000	0·227

Quantitative work and recovery experiments

Saponification. Table 2 shows the recovery of two levels of ubiquinone after saponification, assays being by the borohydride method. Even under these drastic conditions, a mean recovery of 85% was obtained.

Column chromatography. Preliminary experiments showed that chromatography on floridin

earth as practised for vitamin E was unsuitable for ubiquinone. Whereas from some batches of adsorbent nearly quantitative recoveries were obtained, other batches destroyed substantial but varying amounts of the adsorbed ubiquinone, depending on the exact method of column preparation. There is also a considerable hazard in the use of floridin earth as an adsorbent when very small amounts of tocopherols are present in the extract. In the examination of vitamin E-deficient tissues, for example, the total extract from 20 g. of tissue may contain as little as 10 μg. of tocopherol, and, at this level, losses often occur on floridin, whereas such quantities may be safely handled on Decalso F. Table 3 gives the results of recovery experiments with Decalso F. Substances investigated were cholesterol, vitamin A, vitamin K_1, β-carotene, α-tocopherol and ubiquinone 50, in amounts chosen as likely to be encountered during the analysis of 20 g. of animal tissue. After adsorption of the individual substances from light petroleum, the columns were eluted successively with 25 ml. lots of benzene and 5% (v/v) ether in light petroleum (b.p. 40–60°). Both tocopherol and ubiquinone were recovered nearly quantitatively and almost entirely in the benzene fraction. (The volume of benzene was increased to 35 ml. in subsequent analyses.) Small amounts of cholesterol and vitamin A could be removed completely by adsorption but could be eluted with more polar solvent mixtures. Vitamin K_1, at the level employed, could not be recovered. β-Carotene spread over all the fractions, but only a small proportion appeared in the benzene fraction, in an amount that in no way interferes with paper-chromatographic analysis.

Paper chromatography. Table 4 gives the results of experiments to determine the recovery of ubiquinone and ubichromenol. Each substance was chromatographed by the two-dimensional method, the ubichromenol being run together with ubiquinone in order to simulate conditions during a normal assay of animal tissue. Amounts of ubiquinone up to 1 mg. could be chromatographed and recovered in about 90% yield; ubichromenol was recovered quantitatively.

Overall assay recoveries. Although the recovery experiments described above gave satisfactory results, it was important to study more exactly the overall recovery of ubiquinones and ubichromenols to be expected from the complete analysis of animal tissue. The results from such experiments are grouped in Tables 5 and 6. In the first experiment, the kidneys from 12 rats were pooled, quartered and divided into two lots that were separately homogenized. Ubiquinone 50 (1 mg.) was added to

Table 2. *Recovery of ubiquinone 50 after saponification in the presence of pyrogallol*

Amount taken (μg.)	Amount recovered (μg.)	Recovery (%)
250	222	89
1000	800	80

Table 3. *Recovery of ubiquinone, α-tocopherol and some other substances from Decalso F columns*

Each substance was chromatographed in light petroleum (b.p. 40–60°, 25 ml.) and eluted successively with benzene (25 ml.) and 5% (v/v) of ether in light petroleum (25 ml.).

Material	Amount used (μg.)	Amount eluted (μg.)			Total recovery (%)
		Light petroleum	Benzene	Ether–light petroleum	
Cholesterol	500	None	None	None	—
Vitamin A	50	None	None	None	—
Vitamin K_1	200	None	None	None	—
β-Carotene	70	13·5	11·8	11·3	52
α-Tocopherol	260	None	244	8·1	97
Ubiquinone 50	890	None	816	29·2	95

Table 4. *Recovery of ubiquinone and ubichromenol after two-dimensional chromatography*

Substance	Amount chromatographed (μg.)	Amount recovered (μg.)	Recovery (%)
Ubiquinone 50	48·4	45·0	93·1
	96·8	88·2	91·2
	242	215	89·1
	500	455	91·2
	1000	892	89·2
Ubichromenol 45	49·1*	50·7	103

* Co-chromatographed with 460 μg. of ubiquinone 50.

Table 5. *Overall recovery of ubiquinone in tissue analysis, before and after paper chromatography*

1. Rat kidney (pooled) was divided into two parts, A (27·7 g.) and B (19·9 g.). To B, ubiquinone 50 (1 mg.) was added before extraction. A and B were extracted and A was further divided before chromatography on Decalso F; to one half of A, ubiquinone 50 (1 mg.) was added. Determinations were by the borohydride method, without paper chromatography.

2. Rat kidney (pooled, 22·8 g.) was divided into two equal parts. To one half was added ubiquinone 50 (0·86 mg.). The tissues were then assayed by the full method, including paper-chromatographic analysis.

Expt. no.		Control extract	Control + ubiquinone before extraction	Control + ubiquinone before Decalso	Recovery of added ubiquinone (%)
1A	Ubiquinone (μg. in extract)	685	—	1745	106
1B	Ubiquinone (μg./g. of original tissue)	99	141	—	84
2	Ubiquinone (μg./g. of original tissue)	141	209	—	91

Table 6. *Overall recovery of ubiquinone and ubichromenol in vitamin E-deficient rat liver*

Liver (45 g.) was divided into three equal portions: A was assayed, B was taken through the analytical procedure and added to portion C.

Sample	Ubiquinone* (μg./g.)	Ubichromenol (μg./g.)
A	96·2	46·4
B + C	94·6	44·7
Recovery of added substances (%)	98·0	96·1

* Total of three ubiquinones detected.

Table 7. *Effect of light on the extraction of ubiquinone* 50

Ubiquinone was dissolved in acetone (100 ml.) and the solution refluxed on a steam bath for 3 hr. in diffuse daylight and in the dark. The solutions were then evaporated and assayed by the borohydride method.

Conditions	Ubiquinone used (μg.)	Amount recovered (μg.)	Recovery (%)
Light	50·3	40·4	78·2
	212	154	72·6
Dark	50·3	46·5	90·2
	212	189	90·6

Table 8. *Ubiquinone assays before and after paper chromatography*

Sample	Ubiquinone assay (μg./g.) Before paper chromatography	After paper chromatography
Rat liver 1	129·0	96·2
Rat liver 2	94·0	92·0
Rat kidney	74·6	84·9
Keilin–Hartree preparation	1010	1126

one portion and both were then extracted. The extract from the unsupplemented homogenate was now divided into two parts, one of which was supplemented with ubiquinone 50 (1 mg.). The three extracts were then chromatographed on Decalso F and ubiquinone was measured directly in the eluates. In the second experiment, on a different sample of rat kidney, recovery of added ubiquinone 50 was tested through the complete procedure from extraction to paper chromatography. In a third experiment, in which the recovery of ubichromenol was examined, a different technique was used. Rat liver (45 g.), obtained from vitamin E-deficient animals, was homogenized saponified and divided into three equal portions. Two of the three extracts were carried through the complete analysis, but ubiquinone and ubichromenol were only determined in one extract. The final solution of the other extract was added to the third portion of liver, which was then carried through the analytical procedure again. The recoveries of both ubiquinone and ubichromenol are nearly quantitative (Table 6).

Effect of light on ubiquinone analysis. The recoveries of ubiquinone were materially affected if the preliminary extraction of the tissue was carried out in daylight. In Table 7 are shown the losses caused by heating small amounts of ubiquinone 50 in acetone in diffuse daylight.

Comparison of ubiquinone assays before and after paper chromatography

Crane *et al.* (1959) have commented on the existence of a material in heart lipid that shows increased absorption at 275 mμ after addition of borohydride. In our experience, this material is not always removed by the Decalso columns used in the present procedure (depending mainly on the nature of the tissue being analysed). The material appears to exist in several types of animal tissue

Table 9. *Analyses of lipid constituents before and after storage at −20°*

Tissue	Time of storage before analysis (weeks)	α-Tocopherol (μg./g.)	Ubichromenol (μg./g.)	Ubiquinone (μg./g.)
Keilin–Hartree preparation	0	44·8	23·0*	—
	28	42·6	30·8	—
Rabbit liver†	0	7·0	3·7	56·6
	6	6·9	3·9	59·4

* Assay by modified method, with floridin columns.

† From vitamin E-deficient animals.

Table 10. *Vitamin A recovery after homogenization, extraction and saponification*

Sample of rat liver was analysed by two methods. For details, see text.

Sample	Vitamin A (i.u./g. of tissue) Method A	Method B
Liver	1392	1241
Liver + 4120 i.u. of vitamin A palmitate*	—	5320

* Blue value spectroscopic factor 25·4.

Table 11. *Analysis of the Keilin–Hartree preparation*

Substance	Amount (μg./g. of protein)
α-Tocopherol (before reduction–cyclization)	44·8
α-Tocopherol (after reduction–cyclization)	65·2
Ubiquinone	3560
Ubichromenol	23·0
Ubichromenol + RC-ubiquinone (after reduction–cyclization)*	134·0

* Assuming identical spectrophotometric factors for ubichromenol and RC-ubiquinone.

and to be separable by paper chromatography from ubiquinone. Thus, as shown by some typical results in Table 8, in certain assays the ubiquinone determined after paper chromatography is considerably higher than before. A further source of error (also demonstrated by results in Table 8) may be encountered in some tissues; here the ubiquinone assay is considerably higher before paper chromatography than after. Since it has been adequately demonstrated that the recovery of ubiquinone from chromatograms under assay conditions is virtually quantitative, it would seem that there is another type of material remaining in the extract after Decalso which exhibits a change in *E* on borohydride treatment. Since in certain instances it has been observed that the *E* at 275 mμ actually drops to zero after borohydride addition, it is probable that a substance having fluorescence at this wavelength is the cause of the interference. It must be assumed therefore that assays of ubiquinone after Decalso are incorrect if they do not agree with those after paper chromatography.

Effect of storage of tissues at −20°

In a recent paper, Pollard & Bieri (1959) have commented on the variability of *R* values on $ZnCO_3$-treated paper and we agree with their insistence on the removal of sterols before chromatography. It seems that the 'slow' spot described by them is sometimes due to ubichromenol, although the reducing substance of zero migration must be another substance. Pollard & Bieri found an appreciable decrease of 'apparent' tocopherol after storage of tissues at −20°, and, by implication, a corresponding decrease of ubiquinone. In our experience, such losses only occur in tissues that are continually thawed and refrozen for sampling purposes (as may have been the case in the experiments of Pollard & Beiri). As Table 9 shows, we have found that losses of tocopherol, ubichromenol and ubiquinone are negligible over long periods in cold storage.

Vitamin A. A sample of liver prepared from stock-colony rats was divided into three equal parts. One part was analysed for vitamin A by the method (A) of Ames, Risley & Harris (1954). A second part was analysed by the present method (B) of homogenization, extraction and saponification. To the third part was added vitamin A palmitate and the analysis by method (B) repeated. Determinations were by the antimony trichloride reaction in each case. The results (Table 10) show reasonable agreement between the two methods and a 99 % recovery of added vitamin A. No precautions against the effect of light were taken in these experiments.

Analysis of the Keilin–Hartree heart preparation

The sarcosomal horse-heart preparation (Keilin & Hartree, 1940) was extracted by the two extraction procedures, designated A and B by Bouman & Slater (1957) and the extracts were analysed for the lipid constituents by the paper-chromatographic procedures described above. The chromatogram of the untreated extract (procedure A) clearly showed the presence of α-tocopherylquinone, which was

eluted from the paper and identified by its ultraviolet spectrum (peaks at 261 and 271 mμ in ethanol) and by co-chromatography with the pure substance. Ubiquinone, α-tocopherol and ubichromenol were also detected. The chromatogram of the treated extract (B) contained no tocopherylquinone, but a very large quantity of ubiquinone remained. The quantitative data (Table 11) show that only a small amount of the ubiquinone present can be cyclized by the ascorbic acid–HCl procedure, although even the relatively small amount of RC-ubiquinone formed is more than enough to obscure the true cyclized tocopherylquinone if two-dimensional chromatography is not used.

DISCUSSION

The method described for the simultaneous determination of several important lipid substances has been tested on a variety of animal tissues without any particular difficulty appearing. The only modification that is occasionally necessary is during the analysis of certain samples of liver which may have a high vitamin A content. If this is so, the vitamin A may not be retained by 5 g. of Decalso and will run close to α-tocopherol on the paper, where it will be observed as a strongly fluorescent yellow spot. Since vitamin A interferes with the colorimetric determination of tocopherol, this must be avoided by either increasing the size of the column and volume of eluting solvent appropriately or carrying out a separate tocopherol assay and using floridin earth for purification.

It would appear that some sort of paper-chromatographic separation is essential for the precise determination of ubiquinone. The present method seems efficient in that examination of the ultraviolet spectra of ubiquinone spots eluted from the two-dimensional chromatograms of extracts of animal tissues has revealed that in most cases they contain almost pure ubiquinone (the absorption of the small amounts of ubichromenol contributing only slightly to the total absorption). This has been done by comparing the results of the borohydride assay with determinations made from an accurate measurement of the ultraviolet spectra at three points, 265, 274·5 and 282·5 mμ, and comparison with the data from pure ubiquinone.

Recoveries of all the lipid substances have been adequately tested and the use of Decalso columns for purification gives particularly good results with tocopherol and ubichromenol. Although Festenstein, Heaton, Lowe & Morton (1955) and Ward & Moore (1955) found ubiquinone to be alkali-labile in their procedures, the recovery experiments described in the present work indicate that the substance is readily handled in alkali provided that pyrogallol is present. Ubiquinone is, however, light-sensitive and this may account for the results of these earlier workers. There is no need to saponify the whole tissue, which, provided that light is absent, can be subjected to normal lipid extraction.

The present work explains adequately the earlier results of Bouman & Slater (1957) and Bouman *et al.* (1958). It is clear that single-dimensional chromatography on zinc carbonate-treated paper is inadequate for the analysis of animal tissues and, owing to the co-chromatography phenomena we have described, R values can be grossly misleading. Bouman & Slater, by their cyclization procedure, produced the compound we have called RC-ubiquinone, which at first they confused with α-tocopherol. However, the present investigation has shown that their conclusions, i.e. that α-tocopherol and α-tocopherylquinone both exist in the heart preparation, are correct. We have found 44·8 and 20·4 μg./g. of protein respectively of these two substances in the horse-heart preparation; these amounts may well have been concealed by the procedure later described by Bouman *et al.* (1958). There appears to have been some doubt whether α-tocopherylquinone actually exists in tissues. Donaldson & Nason (1957) presumed it was present in their enzyme preparation from rat skeletal muscle but this conclusion was based on the same evidence (acid-cyclization and then chromatography on zinc carbonate-treated paper) as that of Bouman & Slater and is thus unreliable. Neither Draper & Alaupovic (1959) nor Pollard & Bieri (1959) could find the quinone in chick tissues, but Mervyn & Morton (1959) have recently offered positive spectroscopic evidence for its existence in human kidney. Our findings unequivocally support the existence of tocopherylquinone in animal tissue, although so far we have observed it only in the horse-heart preparation. It would not seem possible that quantities of tocopherylquinone of the order found in this preparation could be artifacts. We have never found, during normal laboratory handling, that tocopherols oxidize to give measurable amounts of quinones: degradation, if it occurs, seems usually to proceed beyond the quinone stage. Moreover, analysis of the 'supernatant' from the Keilin–Hartree preparation showed it to contain tocopherol, ubiquinone and ubichromenol in large amounts, but no tocopherylquinone. It would seem therefore that tocopherylquinone is mainly located in those structural components that appear in the final particulate preparation. The quantity observed is very small and this is, no doubt, why examination of whole tissues (where the concentration is relatively much smaller) usually fails to reveal its existence.

There has been a considerable amount of discussion about the presence of vitamin K_1 in animal tissues. Thompson (1957) considers that no direct

chemical evidence exists to indicate the occurrence of vitamin K compounds in animal tissues, and Lester & Crane (1959), who comment on this, looked exhaustively and unsuccessfully for the typical ultraviolet spectrum of vitamin K in their ubiquinone extracts. Nevertheless we have repeatedly observed vitamin K_1 in rat heart (total tissue), the Keilin–Hartree horse-heart preparation and in rat erythrocytes, its identity being confirmed by chromatography and the typical ultraviolet spectrum of the substance. Both quantitatively and qualitatively, however, its observation is uncertain (as indicated by the recovery experiments described above) and obviously depends on modifications of the analytical procedure not subject to control.

SUMMARY

1. A method is given for the simultaneous determinations of tocopherols, ubiquinones and ubichromenols in animal tissues. The substances are separated, after preliminary purification, on a two-dimensional paper-chromatographic system, from which they are eluted and separately measured. Vitamin A and tocopherylquinone can be determined in the same extract.

2. The colorimetric determination of ubichromenol, with ferric chloride and αα′-dipyridyl, is described.

3. The chromatographic behaviour of ubiquinones and ubichromenols has been investigated and conditions are given for their separation.

4. The existence of vitamin K_1 in animal tissue has been confirmed.

5. The Keilin–Hartree horse-heart preparation has been reinvestigated and earlier reports on the presence of α-tocopherol and α-tocopherylquinone have been confirmed.

6. No loss of tocopherol, ubiquinone or ubichromenol was found after prolonged storage of tissues at $-20°$.

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REFERENCES

Ames, S. R., Risley, H. A. & Harris, P. L. (1954). *Analyt. Chem.* **26**, 1378.

Bouman, J. & Slater, E. C. (1957). *Biochim. biophys. Acta*, **26**, 624.

Bouman, J., Slater, E. C., Rudney, H. & Links, J. (1958). *Biochim. biophys. Acta*, **29**, 456.

Boyer, P. D. (1951). *J. Amer. chem. Soc.* **73**, 733.

Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T. (1960). *Biochem. J.* **75**, 460.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Crane, F. L., Lester, R. L., Widmer, C. & Hatefi, Y. (1959). *Biochim. biophys. Acta*, **32**, 73.

Cunningham, N. F. & Morton, R. A. (1959). *Biochem. J.* **72**, 92.

Diplock, A. T., Edwin, E. E., Green, J., Bunyan, J. & Marcinkiewicz, S. (1960). *Nature, Lond.*, **186**, 554.

Donaldson, K. O. & Nason, A. (1957). *Proc. nat. Acad. Sci., Wash.*, **43**, 364.

Draper, H. H. & Alaupovic, P. (1959). *Fed. Proc.* **18**, 218.

Edwin, E. E., Diplock, A. T., Bunyan, J. & Green, J. (1960). *Biochem. J.* **75**, 450.

Festenstein, G. N., Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **59**, 558.

Green, J. (1959). *J. Sci. Fd Agric.* **10**, 801.

Green, J., Marcinkiewicz, S. & Watt, P. R. (1955). *J. Sci. Fd Agric.* **6**, 274.

Kaunitz, H. & Beaver, J. J. (1944). *J. biol. Chem.* **156**, 653.

Keilin, D. & Hartree, E. F. (1940). *Proc. Roy. Soc.* B, **129**, 277.

Laidman, D. L., Morton, R. A., Paterson, J. Y. F. & Pennock, J. F. (1959). *Chem. & Ind.* p. 1019.

Le Rosen, A. L. (1942). *J. Amer. chem. Soc.* **64**, 1905.

Lester, R. L. & Crane, F. L. (1959). *J. biol. Chem.* **234**, 2169.

Lester, R. L., Crane, F. L. & Hatefi, Y. (1958). *J. Amer. chem. Soc.* **80**, 4751.

Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.

Mervyn, L. & Morton, R. A. (1959). *Biochem. J.* **72**, 106.

Morton, R. A., Gloor, U., Schindler, O., Wilson, G. M., Chopard-dit-Jean, L. H., Hemming, F. W., Teler, O., Leat, W. M. F., Pennock, J. F., Rüegg, R., Schweiter, U. & Wiss, O. (1958). *Helv. chim. acta*, **41**, 2343.

Morton, R. A., Wilson, G. M., Lowe, J. S. & Leat, W. M. F. (1957). *Chem. & Ind.* p. 1649.

Pollard, C. J. & Bieri, J. G. (1959). *Biochim. biophys. Acta*, **34**, 420.

Report of the Vitamin E Panel, Society for Analytical Chemistry. Quoted in *Analyst* (1959), **84**, 356.

Rüegg, R., Gloor, U., Goel, R. N., Ryser, G., Wiss, D. & Isler, O. (1959). *Helv. chim. acta*, **42**, 2616.

Thompson, R. H. (1957). *Naturally Occurring Quinones*. New York: Academic Press Inc.

Ward, R. J. & Moore, T. (1955). *Biochem. J.* **59**, xv.

Biochem. J. (1960) **76**, 572

Fission Products and the Dairy Cow

2. SOME ASPECTS OF THE METABOLISM OF THE ALKALINE-EARTH ELEMENTS CALCIUM, STRONTIUM AND BARIUM*

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Radioactive isotopes of strontium (^{89}Sr and ^{90}Sr) are generally regarded as the most dangerous of the products of nuclear fission. The passage of radiostrontium deposited on soil and vegetation into the human diet has therefore received considerable attention. Since strontium is similar to calcium in its metabolic behaviour it has become common practice to study the simultaneous behaviour of the two elements in the various physiological processes in plants and animals which are involved in these transfer processes. In this way it has been demonstrated that discrimination against strontium in favour of calcium occurs, for example, in their passage from the diet of a dairy cow to its milk (Comar & Wasserman, 1958).

^{140}Ba is also a constituent of mixed fission products and is present in significant amounts in tropospheric fall-out but, because of its relatively shorter half-life (12·8 days as compared with 54 days for ^{89}Sr and over 20 years for ^{90}Sr), it is considered to present a smaller hazard to the human and animal population than the strontium isotopes. Distribution studies in human tissues (Sowden, 1958) suggest that the behaviour of ^{140}Ba in the animal body differs from that of strontium and calcium and, in fact, may be more closely related to that of radium.

The present paper describes studies on the comparative behaviour of ^{45}Ca, ^{89}Sr and ^{140}Ba in the dairy cow. These experiments were designed particularly to yield information on the physiological processes involved in the discrimination between the three elements during their transfer from diet to milk.

* Part 1: Garner, Jones & Ekman (1960).

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METHODS

Animals

Mature, lactating Redpoll × Ayrshire cows have been used throughout these experiments. The animals received approx. 30 lb. of hay and commercial dairy nuts (2½ lb./gal. of milk) daily, divided into two equal feeds. Water was freely available. Complete separation of urine and faeces has been achieved by means of the metabolism harness described by Balch, Bartlett & Johnson (1951), modified to facilitate decontamination. Collection of urine and faeces was made at 6 hr. intervals (05.00, 11.00, 17.00 and 23.00 hr. daily) over an experimental period of 8 days. Milking was carried out with a standard machine at 06.00 and 16.00 hr. daily. Blood samples were obtained, by venepuncture, from the mammary veins.

Plan of experiments

Pairs of animals received ^{45}Ca and ^{89}Sr, ^{89}Sr and ^{140}Ba or ^{140}Ba and ^{45}Ca first orally and, 1–2 months later, the same isotopes intravenously. The approximate amounts of activity received by the animals are set out in Table 1.

Administration of radioactivity

In both oral and intravenous experiments, pile-produced, carrier-free ^{89}Sr and an equilibrium mixture of ^{140}Ba and its radioactive daughter ^{140}La in HCl solution neutralized to about pH 6 were used. For oral administration, ^{45}Ca in the form of neutron-irradiated $CaCO_3$ (specific activity approx. 200 μc of ^{45}Ca/g. of Ca) dissolved in the minimum amount of about 5N-HCl was employed. Material of higher specific activity (5–10 mc of ^{45}Ca/g. of Ca) in the form of a solution of $CaCl_2$ was used for intravenous injection.

The large volume of the $CaCl_2$ solution with relatively low specific activity necessitated feeding the material in a standard sheep feed (a mixture of molasses, flaked maize and oats) used on this Station. This procedure was therefore adopted in all the oral experiments. The animals were trained to eat this mixture by being offered small quantities

Table 1. *Plan of experiments*

For details see text.

Animal pair	Date	Treatment
1	16. v. 58	7 mc of ^{45}Ca; 1·5 mc of ^{89}Sr orally
	28. vii. 58	2 mc of ^{45}Ca; 1 mc of ^{89}Sr intravenously
2	6. xi. 58	1·5 mc of ^{89}Sr; 16 mc of ^{140}Ba orally
	3. xii. 58	1·5 mc of ^{89}Sr; 4 mc of ^{140}Ba intravenously
3	12. ii. 59	2 mc of ^{140}Ba; 6 mc of ^{45}Ca orally
	12. iii. 59	1 mc of ^{140}Ba; 3 mc of ^{45}Ca intravenously

for a few days before the experimental period. The large amount of stable Ca inevitably administered had no apparent effect on the blood Ca level. Comar & Wasserman (1956) have also observed that short-term changes in the dietary intake have no effect on Ca metabolism. Intravenous injections were made through an indwelling plastic catheter previously inserted into a mammary vein. A transient increase in blood Ca was observed after these injections. However, the highest value seen in any animal was 18 mg./100 ml. of plasma and values within the normal range were always seen after 1 hr.

The relevant mixture of isotopes was given to each animal immediately after either the morning or afternoon milking.

Counting methods

^{45}Ca and ^{89}Sr experiments. The ^{45}Ca and ^{89}Sr content of faecal samples was estimated by ashing samples (25 g.) of the thoroughly mixed, total daily output of fresh faeces at 650°, placing the ash in aluminium cups of diameter 2·5 cm. and counting under a thin end-window Geiger–Müller counter, type EHM 2 (20th Century Electronics Ltd.) The efficiency of this counting method was approx. 1% for ^{45}Ca and 6% for ^{89}Sr.

Milk from each collection was prepared for counting by pipetting 100 ml. samples into steel dishes of diameter 11·4 cm., adding a few drops of perchloric acid and drying under an infrared lamp. The samples were counted by a β-scintillation counter with a plastic phosphor of diameter 4·5 in., giving an efficiency of 2% for ^{45}Ca and 27% for ^{89}Sr.

Plasma and urine samples (2 and 10 ml. respectively) were assayed by precipitating calcium oxalate and strontium oxalate directly on to steel planchets of diameter 2·4 cm. (Comar, Hansard, Hood, Plumlee & Barrentine, 1951). With urine, carrier Ca (sufficient to give a total precipitate of about 50 mg.) was added to ensure complete precipitation of the radioactivity. Precipitates from both urine and plasma were counted under the end-window Geiger–Müller counter, with efficiencies of approx. 5% for ^{45}Ca and 11% for ^{89}Sr.

The ^{45}Ca and ^{89}Sr were distinguished by counting all samples with and without a polyvinyl chloride filter which absorbed all the ^{45}Ca β-particles but decreased the count rate of ^{89}Sr by 47·7%. The polyvinyl chloride filter was obtained from Velbex sheeting of thickness 0·020 in. and weight 61 mg./cm.^{2}, supplied by BX Plastics Ltd., London, E. 4.

^{89}Sr and ^{140}Ba–^{140}La experiments. Milk and urine were assayed by pipetting samples (100 ml.) into polyethylene bags (6 in. × 5 in.) subsequently sealed by heat. Samples (100 g.) of faeces were weighed into similar bags and the contents flattened, after sealing, into a uniform layer. The bags were counted by placing them flat in a containing tray, situated under the plastic phosphor of the β-scintillation counter. The efficiency of this method was approx. 3% for ^{89}Sr and 4% for ^{140}Ba–^{140}La. Differential counting was accomplished by using a $\frac{1}{8}$ in. Perspex filter, of weight 375 mg./cm.^{2}, which absorbed all the ^{89}Sr β-particles but decreased the count rate due to ^{140}Ba–^{140}La by 66·9%.

Plasma samples were assayed as liquids by means of a Veall-type Geiger-Müller counter. Counts due to ^{140}Ba–^{140}La were determined by counting also in a NaI (Tl) well-type γ-scintillation counter, and surrounding the samples with a thin lead shield to absorb β-particles from ^{89}Sr. The efficiencies of counting were approx. 6·5% for ^{89}Sr and 20% for ^{140}Ba–^{140}La.

^{45}Ca and ^{140}Ba–^{140}La experiments. Faeces samples were prepared for assay by ashing at 650° and dissolving the ash in 20 ml. of 6N-HCl. Calcium oxalate and barium oxalate were precipitated from 2 ml. of this solution on to steel planchets of diameter 2·4 cm. Urine samples (200 ml.) were dried and ashed, the ash was dissolved in 40 ml. of 6N-HCl and the oxalates were precipitated from 5 ml. of this solution. The dried precipitates were counted in the β-scintillation counter. The supernatant solutions from the oxalate precipitations were retained and counted in the Veall-type Geiger–Müller counter as, in some cases, incomplete precipitation of barium oxalate occurred. The proportion of barium unprecipitated was never more than 20%. The efficiencies of counting ^{45}Ca and ^{140}Ba–^{140}La in this way were approx. 8 and 22% respectively.

Plasma and skimmed milk were assayed by pipetting 10 ml. samples into steel dishes of diameter 11·4 cm., drying under an infrared lamp and counting in the β-scintillation counter. (Skimmed milk was used in the later experiments because it could be dried more easily. A correction was made for the cream content of each sample when converting the results into an equivalent concentration in whole milk.) The efficiencies of counting ^{45}Ca and ^{140}Ba–^{140}La in this way were approx. 5·5 and 21% respectively in milk, and 5·7 and 23% respectively in plasma.

The ^{45}Ca and ^{140}Ba–^{140}La were distinguished by means of the polyvinyl chloride filter of weight 61 mg./cm.^{2} This decreased the count from ^{45}Ca to zero but decreased that due to ^{140}Ba–^{140}La by only 63·3%.

In all experiments in which ^{140}Ba–^{140}La was used the samples were set aside for 14 days before counting to allow equilibration between ^{140}Ba and its daughter ^{140}La.

In all the experiments suitable standards were prepared by adding known amounts of the appropriate isotopes to faeces, urine, milk and plasma obtained from the animals before administration of radioactivity. These were treated and counted by the methods already described.

In all experiments sufficient counts were obtained to give a probable error of less than 2% in the determination of each isotope in all samples. Corrections were made for background radiation, the dead-time of the counting instruments used, radioactive decay and, where applicable, self-absorption.

RESULTS

Recovery of radioactivity from faeces, urine and milk

The overall recoveries in the 8 days following administration are given in Table 2.

If it be assumed that the fraction of an orally administered isotope which is absorbed from the gut behaves similarly in every way to the same isotope introduced directly into the blood stream, then

$$\text{Percentage of material absorbed} = 100 \times \frac{\text{Percentage of orally administered material excreted in urine or milk}}{\text{Percentage of intravenously administered material excreted in urine or milk}}.$$

Table 2. *Percentage recovery of alkaline-earth elements from faeces, urine and milk in the 8 days after administration*

Figures given are the mean results (range in parentheses) from four animals.

	Oral administration			Intravenous administration		
	Faeces	Urine	Milk	Faeces	Urine	Milk
^{45}Ca	71·1 (57·2–81·5)	0·39 (0·10–0·64)	16·1 (11·2–23·2)	16·4 12·1–23·8)	1·17 (0·42–2·01)	31·9 (21·2–56·4)
^{89}Sr	89·3 (85·3–94·2)	1·34 (0·76–1·93)	1·94 (1·52–2·61)	18·0 (16·3–20·3)	21·1 (16·4–28·2)	16·4 (10·7–27·0)
^{140}Ba	98·1 (95·4–110·9)	1·08 (0·90–1·34)	0·56 (0·32–0·75)	36·5 (32·1–46·9)	34·2 (20·5–48·4)	10·2 (8·7–13·4)

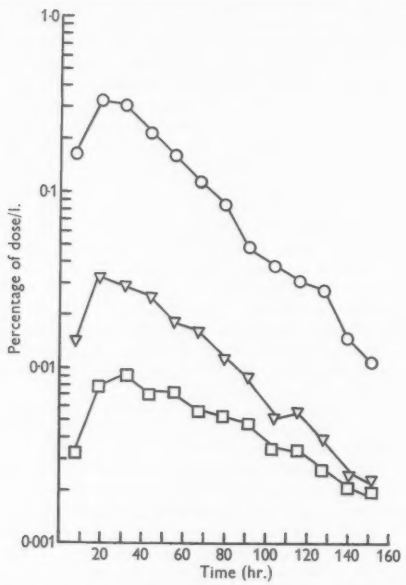

Fig. 1. Change in concentration with time of radioactive alkaline earths in milk after oral administration. ○, ^{45}Ca; ▽, ^{89}Sr; □, ^{140}Ba. Each point has been obtained by averaging the figures for four animals. Correction has been made for physical decay.

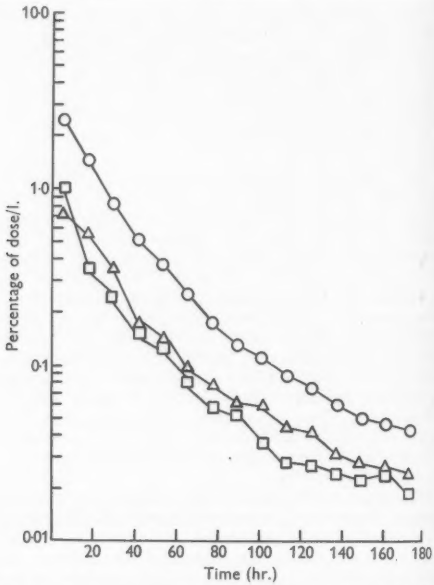

Fig. 2. Change in concentration with time in milk after intravenous administration. ○, ^{45}Ca; △, ^{89}Sr; □, ^{140}Ba. Each point has been obtained by averaging the figures for four animals. Correction has been made for physical decay.

With the results obtained in individual animals after oral administration 34–52 % (mean 40 %) of ^{45}Ca, 8–12 % (mean 11 %) of ^{89}Sr and 4–7 % (mean 6 %) of ^{140}Ba was absorbed from the gut.

Elimination of ^{45}Ca, ^{89}Sr and ^{140}Ba in milk

The change of concentration of these elements with time in milk after oral and intravenous administration is shown in Figs. 1 and 2. Ultimately the rate becomes exponential for each of the isotopes. The biological half-lives, calculated from regression lines fitted through the experimental points on the exponential parts of the curves, after oral administration, can be estimated to be 25, 28 and 53 hr. for ^{45}Ca, ^{89}Sr and ^{140}Ba respectively.

Discrimination between calcium, strontium and barium in their passage from diet to milk

Comar, Wasserman & Nold (1956) introduced the term 'strontium–calcium observed ratio' (OR) to denote the overall discrimination observed in the movement of the two elements from one phase to another under steady-state conditions. The 'observed ratio' can be defined as the product of

a number of 'discrimination factors' (DF), each of which is a measure of the extent to which the physiological process to which it refers contributes to the overall discrimination. Formulae for the derivation of DF's have been given (Comar *et al.* 1956; Wasserman, Lengemann & Comar, 1958), but with no explanation of their bases. An examination of these formulae indicates that, in order that the results of oral experiments may be used for the calculation of DF's, it has been necessary to ignore endogenous secretion into the alimentary canal. As is evident from Table 2, over 30 % of intravenously administered ^{140}Ba is eliminated from the body via this route. It has therefore been considered necessary to derive new formulae which require information from intravenous as well as oral experiments; these expressions are set out in Table 3. They can be shown to reduce to Comar's formulae if endogenous secretion is neglected.

The strontium–calcium $DF_{absorptive}$, i.e. that part of the overall discrimination due to differentiation during intestinal absorption, is defined as the percentage of orally administered strontium absorbed, divided by the percentage of orally administered calcium absorbed. The $DF_{urinary}$ measures the effect urinary excretion has on the relative amounts of strontium and calcium remaining in the body. Similarly, the DF_{faecal} represents the effect of endogenous secretion. The $DF_{lactational\text{-}body}$ is a measure of the effect the mammary gland has in discriminating between the amounts of strontium and calcium eliminated in the milk. The $DF_{lactational\text{-}milk}$ represents the effect that loss of strontium and calcium in the milk has on the amount remaining in the body. The $DF_{lactational\text{-}milk}$ and the $DF_{lactational\text{-}body}$ are obtained from the appropriate OR values (see Comar *et al.* 1956; Wasserman *et al.* 1958). Barium–strontium and barium–calcium discrimination factors are obtained similarly.

Observed ratios and discrimination factors are concepts which are strictly applicable only under steady-state conditions. In order to use the results of single dose experiments for their calculation it is therefore necessary to assume that these results can be used to predict the concentrations which would be found in the excreta under steady-state conditions after continuous administration. Experiments with ^{89}Sr and ^{45}Ca in cattle (Comar & Wasserman, 1956) and goats (Wasserman *et al.* 1958) and with ^{131}I in cattle (R. J. Garner & H. G. Jones, unpublished work) indicate that this assumption is justified. Since the time–concentration curve for repeated administration at equal time intervals is constructed simply by summation from that obtained from single-dose experiments it is apparent that the time to reach a constant concentration in the excreta after continuous administration is essentially the same as the time required for the amount of a single dose appearing in the excreta to fall to a small value. A period of 8 days has been found convenient. After 8 days the concentration in faeces, for example, had fallen to below 2·5 % of the peak value.

Values for OR and DF computed from the formulae in Table 3 and with this method of prediction are given in Table 4.

Table 3. *Expressions used in the calculation of strontium–calcium observed ratios and discrimination factors*

Observed ratios are used to measure overall discrimination between strontium and calcium in their transfer from one phase to another. Discrimination factors measure the contribution of individual physiological processes to this overall discrimination (Comar, Wasserman & Nold, 1956).

$$OR_{sample\text{-}precursor} = \frac{^{89}Sr/^{45}Ca \text{ of sample}}{^{89}Sr/^{45}Ca \text{ of precursor}}$$

$$DF_{absorptive} = \frac{\%\text{ of orally administered } ^{89}Sr \text{ absorbed}}{\%\text{ of orally administered } ^{45}Ca \text{ absorbed}}$$

$$DF_{urinary} = \frac{100 - \%\text{ of intravenously administered } ^{89}Sr \text{ excreted in urine}}{100 - \%\text{ of intravenously administered } ^{45}Ca \text{ excreted in urine}}$$

$$DF_{faecal} = \frac{100 - \%\text{ of intravenously administered } ^{89}Sr \text{ excreted in faeces}}{100 - \%\text{ of intravenously administered } ^{45}Ca \text{ excreted in faeces}}$$

$$DF_{lactational\text{-}milk} = \frac{OR_{milk\text{-}diet}}{DF_{absorptive} \times DF_{urinary} \times DF_{faecal}}$$

$$OR_{body\text{-}diet} = DF_{absorptive} \times \frac{\%\text{ of intravenously administered } ^{89}Sr \text{ retained in body}}{\%\text{ of intravenously administered } ^{45}Ca \text{ retained in body}}$$

$$DF_{lactational\text{-}body} = \frac{OR_{body\text{-}diet}}{DF_{absorptive} \times DF_{urinary} \times DF_{faecal}}$$

Table 4. *Strontium–calcium, barium–strontium and barium–calcium observed ratios and discrimination factors in lactating cows*

Intravenous administration	Sr–Ca	Ba–Sr	Ba–Ca
$OR_{faeces\text{-}injection}$	1·2; 1·3	1·8; 1·9	2·5; 1·9
$OR_{urine\text{-}injection}$	18; 11	1·8; 2·1	33; 91
$OR_{milk\text{-}injection}$	0·46; 0·48	0·81; 0·92	0·41; 0·47
Oral administration			
$OR_{faeces\text{-}diet}$	1·5; 1·2	1·1; 1·1	1·4; 1·1
$OR_{urine\text{-}diet}$	4·6; 2·5	0·75; 1·7	2·2; 13
$OR_{milk\text{-}diet}$	0·11; 0·11	0·33; 0·30	0·05; 0·06
$DF_{absorptive}$	0·2; 0·3	0·3; 0·5	0·1; 0·2
$DF_{urinary}$	0·7; 0·8	0·8; 0·8	0·8; 0·6
DF_{faecal}	1·0; 1·0	0·8; 0·8	0·8; 0·70
$DF_{lactational\text{-}milk}$	0·6; 0·5	1·4; 1·1	0·7; 0·7
$OR_{body\text{-}diet}$	0·17; 0·34	0·18; 0·13	0·09; 0·02
$DF_{lactational\text{-}body}$	1·0; 1·5	0·8; 0·5	1·2; 0·3

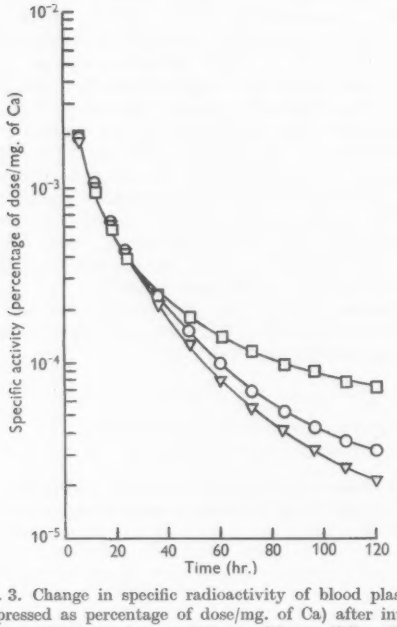

Fig. 3. Change in specific radioactivity of blood plasma (expressed as percentage of dose/mg. of Ca) after intravenous administration. ○, ^{45}Ca; ▽, ^{89}Sr; □, ^{140}Ba. Each point has been obtained by averaging the figures for four animals. Correction has been made for physical decay.

Skeletal metabolism of ^{45}Ca, ^{89}Sr *and* ^{140}Ba

Bauer, Carlsson & Lindquist (1955*a*) have described methods for differentiating quantitatively between skeletal exchange reactions, accretion and resorption, and have applied these methods to a comparative study of the metabolism of ^{90}Sr and ^{45}Ca and of ^{140}Ba and ^{45}Ca in the young rat (Bauer, Carlsson & Lindquist, 1955*b*, 1956).

Table 5. *'Accretion rate' and 'exchangeable calcium' in the whole body of cows*

Calculated from	'Accretion rate' (g. of Ca/hr.)	'Exchangeable Ca' (g.)
^{45}Ca	1·4	41
^{89}Sr	1·3	39
^{140}Ba	0·9	33

Initially attempts were made to apply these methods with, as bone samples, caudal vertebrae removed at intervals after intravenous administration of the isotopes. It became apparent, however, that the distribution of ^{45}Ca and ^{89}Sr was not uniform throughout the length of the tail and the results reported here are therefore confined to 'accretion rates', etc. in the whole skeleton, the whole body being treated in the same manner as a calcified tissue (see Bauer *et al.* 1955*a*). The total percentage of activity remaining in the body at any time was obtained by subtracting from 100 the percentage of the dose eliminated at that time in the urine, faeces and milk.

The change in activity in the blood plasma after intravenous injection is shown in Fig. 3. To facilitate comparison of the behaviour of the three isotopes the 'specific activity' in each case has been expressed as the percentage of the dose/mg. of Ca.

The 'accretion rate' and 'exchangeable calcium' are given in Table 5. These have been calculated by the formulae given by Bauer *et al.* (1955*a*) together with the mean values from four animals for percentage dose retained ($^{45}Ca_{Obs}$, $^{89}Sr_{Obs}$, $^{140}Ba_{Obs}$) and specific activity 9 and 21 hr. after dosing.

With these constants, curves have been constructed showing the changes with time of the amount of 'exchangeable' ^{45}Ca, ^{89}Sr or ^{140}Ba ($^{45}Ca_E$ etc. in the notation of Bauer *et al.* 1955*a*),

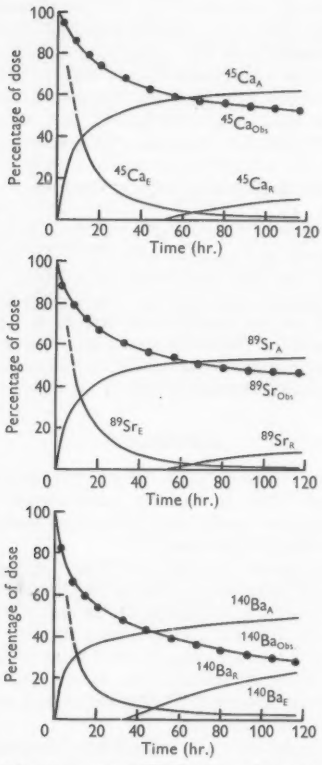

Fig. 4. Overall metabolism in the whole body (mean results for four animals). $^{45}Ca_{Obs}$, $^{89}Sr_{Obs}$, $^{140}Ba_{Obs}$: percentage of intravenously administered ^{45}Ca, ^{89}Sr or ^{140}Ba retained in the whole body. $^{45}Ca_E$, $^{89}Sr_E$, $^{140}Ba_E$: exchangeable fraction of ^{45}Ca, ^{89}Sr or ^{140}Ba in whole body. $^{45}Ca_A$, $^{89}Sr_A$, $^{140}Ba_A$: non-exchangeable fraction of ^{45}Ca, ^{89}Sr or ^{140}Ba in whole body. $^{45}Ca_R$, $^{89}Sr_R$, $^{140}Ba_R$: ^{45}Ca, ^{89}Sr or ^{140}Ba resorbed ($Ca_R = Ca_A + Ca_E - Ca_{Obs}$ etc.). The notation used is that of Bauer, Carlsson & Lindquist (1955*a*).

the amount incorporated into the 'non-exchangeable' fraction of the skeleton ($^{45}Ca_A$ etc.) and the amount removed as a result of resorption ($^{45}Ca_R$ etc.) (Fig. 4).

DISCUSSION

The most striking differences in the behaviour of ^{45}Ca, ^{89}Sr and ^{140}Ba are in the relative amounts eliminated from the body by the various routes after intravenous administration (Table 2). The ability of the ruminant kidney to differentiate between calcium, strontium and barium is particularly noteworthy and is mentioned below in connexion with individual discriminating processes. Comparison of the patterns of excretion of the three isotopes in the cow after intravenous administration with those in a typical non-ruminant animal such as the rat (Bauer *et al.* 1955*b*, 1956) indicates that the relative importance of the faecal as compared with the urinary route is greater in the ruminant than in the non-ruminant animal with calcium, approximately the same with strontium and considerably less with barium.

The time course of secretion into milk, after oral dosing, agrees closely with published results both for ^{45}Ca (Visek, Monroe, Swanson & Comar, 1953) and ^{89}Sr (Squire, Middleton, Sansom & Coid, 1958; Garner & Sansom, 1959). The 'tailing off' of the secretion curve after 6–7 days observed in other experiments is not apparent from Fig. 1, but presumably the biological half-lives of the concentration of the two isotopes in milk would ultimately become identical with those in bone. The longer half-life of ^{140}Ba in milk probably reflects the more rapid release of this isotope from the skeleton (see below).

It is seen from Fig. 1 that, during their transfer from diet to milk, there is considerable discrimination between calcium, strontium and barium in favour of calcium. In this series of experiments ^{45}Ca in the diet was preferentially transferred to the milk by a factor of 9 over strontium and of 18 over barium. The strontium–calcium $OR_{milk\text{-}diet}$ observed is in agreement with values reported by Comar & Wasserman (1958) and Cragle & Demott (1959).

It is apparent from Table 4 and by comparison of Figs. 1 and 2 that absorption from the gut plays the major part in this overall discrimination. Absorptive discrimination is particularly effective in differentiating between calcium and barium (mean $DF_{absorptive}$ 0·16). Mammary differentiation is second in importance with strontium and calcium but, with barium and calcium, the effects of urinary excretion, mammary secretion and endogenous secretion into the gut are almost equal.

Renal discrimination is apparently more complete in ruminants than in non-ruminants. The strontium–calcium $OR_{urine\text{-}blood}$ has been found to be 5 in the rat, for example (Comar *et al.* 1956), 12–20 in the goat (Wasserman *et al.* 1958), and 11–18 in the cow (present paper). The barium–calcium $OR_{urine\text{-}injection}$ after intraperitoneal administration of ^{45}Ca and ^{140}Ba to rats can be estimated to be about 10 (Bauer *et al.* 1956), as compared with a value of 30–90 after intravenous injection in the cow. Because the amount of calcium in ruminant urine is normally small, the effect of the kidneys on overall discrimination, as indicated by the $DF_{urinary}$ (Table 4), is only slightly greater than that in the non-ruminant animal.

Endogenous secretion into the gut is usually considered to play only a minor part in overall discrimination between strontium and calcium (Comar & Wasserman, 1958), although Cragle & Demott (1959) found an increase in the ^{89}Sr–^{45}Ca ratio of faeces with time, after simultaneous oral administration of the two isotopes, which they consider to be suggestive of some preferential secretion of strontium. The present results indicate that differential excretion via this route is small when strontium is considered but is of greater importance for barium. From the results of Bauer *et al.* (1956) a barium–calcium $OR_{faeces\text{-}injection}$ in the rat of about 3 can be inferred, indicating that 'faecal' discrimination is of the same general order in both ruminant and non-ruminant animals.

Despite the operation of a mechanism (mammary secretion) which tends to increase the strontium–calcium ratio in the body ($DF_{lactational\text{-}body} > 1$), the $OR_{body\text{-}diet}$ in the lactating cow (mean value 0·25) is of the same order as, or possibly slightly less than, that recorded in non-lactating, non-ruminant animals (0·16–0·54) (Comar & Wasserman, 1958).

The values for strontium–calcium OR's and DF's obtained in the present series of experiments are in general agreement with those published by Wasserman *et al.* (1958) for the goat.

The validity of the assumptions made by Bauer *et al.* (1955*a*) in deriving expressions for obtaining the accretion rate of calcium in bone and the exchangeable fraction of calcium in bone is open to question; it has, for example, been pointed out by Marshall, Rowland & Jowsey (1959) that there are long-term exchange reactions in bone, and these are not considered by Bauer *et al.* Nevertheless, these parameters are useful for comparing the behaviour of the different bone-seeking isotopes in any one species or of any one of them in different species. Unfortunately a direct comparison of the metabolism of ^{45}Ca, ^{89}Sr and ^{140}Ba in the young rats used by Bauer and his collaborators and the mature cows used in the present experiments is precluded by the difference in the ages of the two groups of animals.

The overall retention curve ($^{45}Ca_{Obs}$, $^{89}Sr_{Obs}$, $^{140}Ba_{Obs}$ of Fig. 4), if plotted on semi-logarithmic paper, can be resolved into three exponential components of half-life 5·5, 20·9 and 1085 hr., 5·8, 18·6 and 1083 hr., and 3·7, 21·6 and 199 hr. respectively for the three isotopes.

The rate of removal of ^{89}Sr and ^{45}Ca from the plasma of cows by exchange reactions ('exchangeable calcium') appears to be approximately the same; the rate of removal of ^{140}Ba may be smaller (Table 5). Bauer *et al.* (1955*b*, 1956) found that, in rats, there was little difference between the behaviour of the three isotopes in this respect. Whereas in rats the rate of incorporation of strontium into the non-exchangeable fraction of the bone-salt was the same as that of calcium (Bauer *et al.* 1955*b*) and that of barium twice that of calcium (Bauer *et al.* 1956), in cows the 'accretion rate' for strontium is 91 % and that for barium 61 %, of that for calcium. ^{140}Ba appears to be released (by resorption) from bone after a shorter time interval and more rapidly than either ^{45}Ca or ^{89}Sr (Fig. 4).

The important overall effect of the differences in metabolism of ^{45}Ca, ^{89}Sr and ^{140}Ba is that a considerably smaller proportion of dietary ^{89}Sr or ^{140}Ba than of dietary ^{45}Ca appears in the milk (Fig. 1) and is retained in the body (Fig. 4). Qualitatively, ^{140}Ba shows marked differences in its behaviour in the animal body from the other alkaline-earth elements studied.

SUMMARY

1. The isotopes ^{45}Ca and ^{89}Sr, ^{89}Sr and ^{140}Ba, or ^{45}Ca and ^{140}Ba have been administered simultaneously, first orally and, later, intravenously, to pairs of lactating cows.

2. The mean recoveries of ^{45}Ca, ^{89}Sr and ^{140}Ba, in the 8 days after oral administration, were (respectively): from faeces, 71, 89 and 98 %; from urine, 0·4, 1·3 and 1·1 %; from milk, 16, 1·9 and 0·6 % of the dose; and, in the 8 days after intravenous administration: from faeces, 16, 18 and 36 %; from urine, 1·2, 21 and 34 %; from milk, 32, 16 and 10 % of the dose.

3. Calculation of individual discrimination factors, by formulae which require information from intravenous, in addition to oral, experiments, indicates that absorptive discrimination made the most important contribution to overall discrimination between calcium, strontium and barium during their passage from diet to milk. Mammary secretion also played a considerable part with strontium; but, with barium, the effects of renal excretion, endogenous secretion into the gut and mammary secretion were approximately equal.

4. The comparative behaviour of ^{45}Ca, ^{89}Sr and ^{140}Ba in the whole body treated as a calcified tissue has been examined. The rate of removal of ^{45}Ca and ^{89}Sr from plasma by exchange reactions was approximately the same; the rate of removal of ^{140}Ba by this process was 80 % of that of ^{45}Ca. The rates of incorporation of ^{89}Sr and ^{140}Ba into the non-exchangeable fraction of the whole skeleton were 91 and 61 % respectively of that of ^{45}Ca. ^{140}Ba was released from the skeleton after a shorter time interval and at a greater rate than ^{45}Ca or ^{89}Sr.

5. The metabolism of ^{45}Ca, ^{89}Sr and ^{140}Ba in the cow is discussed in relation to published results on a typical non-ruminant animal, the rat.

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REFERENCES

Balch, C. C., Bartlett, S. & Johnson, V. W. (1951). *J. agric. Sci.* **41**, 98.

Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1955*a*). *K. fysiogr. Sällsk. Lund Förh.* **25**, no. 1.

Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1955*b*). *Acta physiol. scand.* **35**, 56.

Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1956). *Biochem. J.* **63**, 535.

Comar, C. L., Hansard, S. L., Hood, S. L., Plumlee, H. P. & Barrentine, B. F. (1951). *Nucleonics*, **8**, 19.

Comar, C. L. & Wasserman, R. H. (1956). In *Progress in Nuclear Energy. Series VI. Biological Sciences*, vol. 1. London and New York: Pergamon Press Ltd.

Comar, C. L. & Wasserman, R. H. (1958). In *Radioisotopes in Scientific Research*, vol. 4. London, New York and Paris: Pergamon Press Ltd.

Comar, C. L., Wasserman, R. H. & Nold, M. M. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 859.

Cragle, R. G. & Demott, B. J. (1959). *J. Dairy Sci.* **42**, 1367.

Garner, R. J., Jones, H. G. & Ekman, L. (1960). *J. agric. Sci.* (in the Press).

Garner, R. J. & Sansom, B. F. (1959). *Vet. Rec.* **71**, 670.

Marshall, J. H., Rowland, R. E. & Jowsey, J. (1959). *Radiat. Res.* **10**, 258.

Sowden, E. M. (1958). *Biochem. J.* **70**, 712.

Squire, H. M., Middleton, L. J., Sansom, B. F. & Coid, C. R. (1958). In *Radioisotopes in Scientific Research*, vol. 4. London, New York and Paris: Pergamon Press Ltd.

Visek, W. J., Monroe, R. A., Swanson, E. W. & Comar, C. L. (1953). *J. Dairy Sci.* **36**, 373.

Wasserman, R. H., Lengemann, F. W. & Comar, C. L. (1958). *J. Dairy Sci.* **41**, 812.

Biochem. J. (1960) **76**, 579

Uncoupling Reagents and Metabolism

1. EFFECTS OF SALICYLATE AND 2:4-DINITROPHENOL ON THE INCORPORATION OF ^{14}C FROM LABELLED GLUCOSE AND ACETATE INTO THE SOLUBLE INTERMEDIATES OF ISOLATED RAT TISSUES

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Salicylate uncouples oxidative-phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956; Penniall, Kalnitsky & Routh, 1956; Jeffrey & Smith, 1959; Packer, Austen & Knoblock, 1959). The increased oxygen consumption and diminished amounts of adenosine triphosphate and creatine phosphate observed in the isolated rat diaphragm incubated with salicylate (Smith & Jeffrey, 1956) are directly explicable in terms of this uncoupling action. However, several other reported effects of salicylate on the metabolism of isolated tissues, e.g. the decreased glycogen and protein synthesis produced in rat-liver slices and in diaphragm muscle (Smith, 1955; Manchester, Randle & Smith, 1958), bear a less obvious relationship to uncoupling and could be caused by salicylate acting on enzyme systems other than those involved in oxidative phosphorylation.

The present study is concerned with the effects of salicylate and 2:4-dinitrophenol on the incorporation of ^{14}C from [^{14}C]glucose and [2-^{14}C]acetate into the soluble metabolic intermediates of preparations of isolated rat tissues. The work was undertaken specifically to determine if salicylate produced any effects differing from those of the classical uncoupling agent and more generally to investigate the wider implications of an uncoupling action on tissue metabolism. A preliminary account of this work has been previously published (Smith & Moses, 1960).

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EXPERIMENTAL

Preparation of tissues. Male rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet no. 41, were starved for 24 hr. before being killed by stunning and decapitation. The required tissue (liver, kidney, brain, heart muscle or testis) was removed and placed in ice-cold medium. This medium [based on that used by Randle & Smith (1958) except that the K:Na ratio was altered according to the suggestion of Hastings, Teng, Nesbett & Sinex (1952)] contained (m-moles/l.): K_2HPO_4, 10·1; KCl, 123·0; NaCl, 4·5; Na_2SO_4, 0·3; $CaCl_2$, 1·35; $MgCl_2$, 1·3; glucose 1·0, dissolved in de-ionized water and adjusted to

pH 7·4 by the addition of HCl. All the tissues, except testis, were then cut into slices nominally 0·728 mm. thick, with a tissue chopper (McIlwaine & Buddle, 1953). The slices were subsequently cut in two directions at intervals of 0·312 mm., to form small blocks. The consistency of testis, after the removal of its capsule, was such that the preliminary slicing procedure was omitted. The approximate wet weights of the tissues initially used for chopping were: liver, 3·5 g.; kidney, 2·0 g.; brain, 1·8 g.; heart muscle, 1·3 g.; testis, 3·9 g. The chopped tissue was suspended in 15 ml. of the medium, centrifuged for 30 sec. at about 90 *g* and the supernatant containing the tissue debris was discarded. This procedure was repeated with the deposit and the tissue was finally resuspended in 10 ml. of medium. All these operations, including the slicing of the tissues, were performed in a room maintained at 0–2°. A sample (2 ml.) of the final tissue suspension was washed several times with water and dried to constant weight at 105° in a tared tube for the determination of the dry weight.

Incubation conditions. Samples of the final tissue suspension (1 ml.) were dispensed from a wide-mouthed pipette into each of six 25 ml. Erlenmeyer flasks. Sodium salicylate or 2:4-dinitrophenol (DNP) solution (0·05 ml.) was added to produce final concentrations of 5 mM-salicylate or 0·5 mM-DNP. In the controls, water was added instead of the uncoupler. A solution of radioactive substrate (0·01 ml.) was then added as either [^{14}C]glucose (10 μc, 0·4 μmole) or [2-^{14}C]acetate (10 μc, 2 μmoles). The ^{14}C-labelled material were obtained from The Radiochemical Centre, Amersham, Bucks. The mixtures were incubated in an atmosphere of air for 3 hr. at 37° in a Dubnoff metabolic shaking machine (H. Mickle, Mill Works, Gomshall, Surrey). Water (5 ml.) was added to each flask 5 sec. before the end of the incubation period, at which time the tissue was separated from the medium by rapid filtration under reduced pressure through a sintered-glass funnel (30 mm. diam. disk; pore size 40–90 μ) containing a layer of Celite filter aid about 1 mm. thick. The reaction flask and tissue were then washed with a further 5 ml. of water and the funnel, which had a rubber stopper attached to its stem, was removed from the filtration flask and inserted into a centrifuge tube so that the rubber stopper formed a seal. Boiling aqueous 80% (v/v) ethanol (5 ml.) was then poured on to the sintered disk to kill the tissue. About 12 sec. elapsed between filtration of the incubation mixture and the addition of the boiling ethanol. The tissue was allowed to remain in contact with the ethanol for 1 hr. and, after the rubber stopper in the centrifuge tube had been loosened, the ethanolic extract was forced through the filter by compressed air. The funnel was reseated in the centrifuge tube and the tissue further extracted with 2 ml. of boiling 20% (v/v) ethanol, followed by 2 ml. of boiling water. These extracts were combined and evaporated under reduced pressure at 40° to a final volume of 0·6 ml. The filtrate and washings from the original separation of the tissue, immediately after incubation, were also combined and made up to a volume of 15 ml. with absolute ethanol.

Chromatography. The radioactive substances in the concentrated cell extracts and in the filtered medium were separately analysed by two-dimensional descending paper chromatography, 46 cm. × 57 cm. sheets of Whatman no. 4 filter paper, washed with 1% (w/v) oxalic acid, being used. Phenol–water (72%, w/w) was employed as the first solvent and butanol–propionic acid–water (45·9:23·4:30·6, by vol.) as the second (Benson *et al.* 1950). Of the 0·6 ml. of concentrated cell extract obtained in each experiment, 0·2 ml. was chromatographed for 8–10 hr. until the solvents reached the edges of the paper. This chromatogram contained all the substances soluble in aqueous ethanol present in the extract but the various phosphate esters had relatively low R_F values and tended to remain near the origin. The remaining 0·4 ml. of the extract was therefore chromatographed for 24 hr. in each solvent to ensure a more complete separation of the phosphate esters. The high salt content of the filtered medium restricted the quantity which could be analysed by paper chromatography, and 0·5 ml. of the total 15 ml. volume was used, the solvents being allowed to run only to the edges of the paper. The radioactive substances on the chromatogram were located by radioautography with Kodirex X-ray film (Kodak Ltd.). The radioactivity in each spot was counted directly on the chromatogram for 1 min. by means of a Scott type Geiger–Müller tube (Fuller, 1956), flushed continuously with helium which had previously been passed through ice-cold ethanol. Both sides of the chromatogram were counted and the values averaged. The accuracy of each count was approximately proportional to the square root of the number of disintegrations recorded.

The radioactive spots on the chromatogram were identified presumptively by their chromatographic positions and this was confirmed by cochromatography with authentic materials in the same solvent system (Benson *et al.* 1950; Moses, 1960). Phosphate esters were hydrolysed with human prostatic acid phosphatase, prepared by centrifuging the cells from semen and using the supernatant as a source of the enzyme (Schmidt, 1955), and rechromatographed to identify the organic components.

RESULTS

Total incorporation of ^{14}C

The radioactivity measured was that present in the soluble intermediates of the cell extract and medium. The ^{14}C evolved as CO_2 or incorporated into substances, such as glycogen and proteins, which were insoluble in aqueous ethanol, was not estimated. The fate of all the isotope originally present in the labelled glucose or acetate could not therefore be followed. However, an approximate balance of the ^{14}C from the labelled glucose was made by determining the amount of radioactivity remaining as glucose at the end of the experiments. A similar procedure for the [2-^{14}C]acetate was not feasible because of its volatility during chromatography. Brain and testis were found to incorporate 35% of the total ^{14}C from the labelled glucose into the soluble intermediates, leaving 5% as unchanged substrate. Thus 60% of the ^{14}C was liberated as CO_2 or entered ethanol-insoluble substances. Percentages of the ^{14}C incorporated into the soluble intermediates by kidney, heart and liver were 20, 14 and 2 respectively, the remaining isotope being almost completely recovered as unchanged glucose.

The total carbon from the labelled glucose and acetate which was incorporated into the soluble intermediates of the five tissues is given in Table 1.

The least active tissues, liver and kidney, showed proportionally larger incorporations of carbon from acetate than from glucose.

The results in Table 2 show that 5 mM-salicylate and 0·5 mM-DNP inhibited the total incorporation of ^{14}C from the labelled acetate to a much larger extent than that from the labelled glucose. This inhibition was more pronounced for those tissues, liver and kidney, which showed a relatively low glucose:acetate ratio (cf. Table 1). DNP caused some decrease in the incorporation of ^{14}C from [^{14}C]glucose in liver and kidney, whereas both salicylate and DNP decreased the isotope incorporation in testis but increased it in the brain preparation.

Table 1. *Incorporation of carbon from glucose and acetate into the soluble intermediates of rat tissues*

The tissues were cut into blocks and incubated aerobically for 3 hr. at 37° with [^{14}C]glucose or [2-^{14}C]acetate of known specific activities. The unlabelled glucose present in the medium was taken into consideration when calculating the specific activity of the [^{14}C]glucose. The tissues were killed and extracted with ethanol, the extracts were chromatographed and radioactivity was measured in the separated metabolites. From the quantities of ^{14}C incorporated into the metabolites, the total utilizations of glucose and acetate were calculated and are expressed below as μmoles of substrate incorporated into the extract of 25 mg. of dry tissue in 3 hr. The values are calculated from the sum of the ^{14}C present in the metabolites in the cell extract and in the medium.

Tissue	Glucose utilization (μmoles)	Acetate utilization (μmoles)	Glucose utilization / Acetate utilization
Liver	0·07	0·3	0·23
Kidney	0·57	2·0	0·29
Heart	0·50	0·1	5·0
Brain	1·21	0·2	6·1
Testis	2·90	1·1	2·6

Table 2. *Effects of salicylate and 2:4-dinitrophenol on the total incorporation of ^{14}C from [^{14}C]glucose and [2-^{14}C]acetate into the soluble intermediates of rat tissues*

Experimental details were as given in Table 1; salicylate concentration, 5 mM; DNP concentration, 0·5 mM. Results are expressed as percentage inhibitions of ^{14}C incorporations compared with the values observed in the absence of the uncoupling reagents given in Tables 3 and 4. Minus signs indicate stimulation of ^{14}C incorporation.

	[^{14}C]Glucose		[2-^{14}C]Acetate	
Tissue	Salicylate	DNP	Salicylate	DNP
Liver	0	18	92	92
Kidney	1	15	93	91
Heart	0	2	32	32
Brain	−26	−22	70	63
Testis	17	40	60	41

Distribution of ^{14}C in the soluble intermediates

The results in Tables 3 and 4 show the amounts of ^{14}C incorporated into the soluble intermediates of the five tissues in the presence and absence of salicylate and DNP.

In the absence of the inhibitors the general pattern of incorporation from both substrates was similar for the five tissues although there was some variation in the percentage distributions of radioactivity in the various fractions. The ^{14}C from the labelled glucose was found in a number of substances known to be involved in established metabolic sequences. The occurrence of radioactivity in the hexose phosphates, phosphoglyceric acid, phosphoenolpyruvic acid and lactic acid is evidence of the glycolytic pathway, in phosphogluconic acid, ribose and sedoheptulose monophosphates is evidence of the hexose monophosphate oxidative sequence and in fumaric acid, malic acid and citric acid is evidence of the tricarboxylic acid cycle. Radioactivity was also detected in various amino acids (glutamic and aspartic) which are derived from α-oxoglutarate and oxaloacetate by transamination reactions. Small amounts of ^{14}C were also detected in adenosine and inosine nucleotides and in uridine diphosphoglucose. Radioactive maltose could not be detected in brain, which incorporated a high proportion of the isotope into the amino and organic acids. Similar results with brain preparations incubated with [^{14}C]glucose have been reported by Chain (1959). The ^{14}C from the labelled acetate appeared mainly in the amino and organic acids but kidney and liver showed radioactivity in the sugar phosphates, phosphoglyceric acid and phosphoenolpyruvic acid and in inosine phosphates. The results were similar to those described by Katz & Chaikoff (1955).

The distribution of ^{14}C from the labelled acetate was much more affected by salicylate and DNP than that from the labelled glucose. The incorporation of the isotope from the acetate was decreased in all the fractions of the soluble intermediates and, when present, was restricted to the amino and organic acids. Relatively little change was observed with the [^{14}C]glucose and there was considerable variation between the tissues. Kidney (Table 3) showed a decreased incorporation of ^{14}C into sugar phosphates, maltose, uridine diphosphoglucose and nucleotides, with no appreciable changes in the amino and organic acids. The increased total incorporation of ^{14}C observed with brain in the presence of the uncoupling reagents

Table 3. *Metabolism of* [^{14}C]*glucose and* [2-^{14}C]*acetate by rat liver and kidney in the presence and absence of salicylate and* 2:4-*dinitrophenol*

Each flask contained 1 ml. of tissue suspension (24·2 mg. dry wt. of liver or 12·3 mg. dry wt. of kidney) in the presence and absence of either 5 mM-salicylate or 0·5 mM-DNP. The tissue was incubated with 10 μC of labelled substrate for 3 hr. at 37°. The table shows the ^{14}C present in each substance in the whole sample, and thus represents the sum of the ^{14}C present in the cell extract and in the medium. Radioactivity is expressed as 10^{-3} × counts/min. per 25 mg. dry wt. of tissue.

	Liver						Kidney					
Labelled substrate	[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate		
Uncoupling reagent	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP
Sugar diphosphates*	1·4	0·2	0·2	0	0	0	5·5	0·4	0·4	0	0	0
Sugar monophosphates†	6·3	2·2	3·4	0·2	0	0	31	28	16	0·4	0	0
Phosphogluconic acid	0·2	0	0·1	0	0	0	0·2	0	0	0	0	0
Phosphoglyceric acid	1·5	0·3	0·1	2·9	0·2	0·2	3·7	0·6	1·0	0·6	0	0
Phosphoenolpyruvic acid	0·2	0·1	0·05	0·05	0	0·05	1·8	4·9	0·6	0·2	0	0
Uridine diphosphoglucose	1·1	0·1	0·1	0	0	0	3·5	0·6	0·4	0·2	0	0
Adenosine diphosphate	0·5	0·1	0	0	0	0	0·8	0	0	0	0	0
Adenosine triphosphate	0·1	0	0·1	0	0	0	1·6	0	0	0	0	0
Inosine monophosphate	0·2	0	0	0	0	0	0	0	0	0	0	0
Inosine and guanosine diphosphate	0·2	0	0	0·9	0	0	0·2	0	0	0·2	0	0
Inosine triphosphate	0	0	0	0	0	0	0·4	0	0	0	0	0
Maltose	5·1	3·5	6·2	0	0	0	15	2·8	2·8	0	0	0
Fructose‡	4·9	0	5·5	0	0	0	0	42	0	0	0	0
Cysteine	0·3	0	0	0	0	0	0	0	0	0	0	0
Aspartic acid	7·4	8·4	10	12	0·7	0·8	47	35	41	140	11	8·7
Glutamic acid	0·4	0·4	0	18	0·2	0·2	61	28	48	480	33	34
Alanine	13	9·8	15	6·7	0·9	0·8	170	130	100	200	6·7	16
Histidine	0	0	0	1·0	0·2	0	0	0	0	0	0	0
Threonine	0	0	0	1·1	0·7	1·9	0	0	0	44	0	0
Glutamine	0	0	0	6·7	0	0	0	0	0	0	0	0
γ-Aminobutyric acid	0	0	0	1·0	3·1	0	0	0	0	9·4	0·2	0
Glycine + serine§	—	—	—	34	1·2	0	—	—	—	3·9	0	0
Citrulline	0	0	0	0·1	0·3	0·1	0	0	0	0	0	0
Phenylalanine + leucine‖	3·4	7·5	13	1·5	0	3·9	9·6	0	0	0	0	0
Glutathione	0	0	0	0	0	0	0·4	0·4	0	0	0	0
Valine‖	0	1·7	2·3	12	0	0	9·1	49	38	0	0	0
Lactic acid¶	0·6	2·7	0·2	40	0·5	1·8	17	20	28	47	6·9	8·7
Fumaric acid	0	0	0	0·3	0·2	0·2	0·2	0·2	0	0·6	0	0·6
Malic acid	0	0	0	0·7	1·5	0·4	2·8	6·9	9·6	67	4·5	14
Citric acid	0	0	0	0·1	0·1	0·3	0·6	0·6	4·5	0·6	0·4	0·8
Glycollic acid	0	0	0	0	0·9	0·8	0	0	0	0	0	0
Total in:												
Sugar phosphates	9·6	2·8	3·8	3·1	0·2	0·3	42	34	18	1·0	0	0
Uridine diphosphoglucose	1·1	0·1	0·1	0	0	0	3·5	0·6	0·4	0·2	0	0
Nucleotides	1·0	0·1	0·1	1·0	0	0	3·0	0	0	0·2	0	0
Maltose	5·1	3·5	6·2	0	0	0	15	2·8	2·8	0	0	0
Amino acids	24	28	40	95	7·3	7·7	300	250	240	880	52	65
Organic acids	0·7	2·7	0·2	41	3·2	3·4	20	28	42	120	12	24
Unidentified compounds	7·0	2·1	3·5	16	1·0	1·5	23	45	52	25	13	12
Soluble fraction	54	39	59	160	12	13	400	400	350	1000	75	94

* Contains diphosphates of fructose and glucose.
† Contains monophosphates of glucose, fructose, ribose and sedoheptulose.
‡ Possibly a contaminant in the [^{14}C]glucose used as substrate. Its appearance as a separate spot may depend on a variable separation from the residual glucose.
§ Not separated chromatographically. As the two amino acids have chromatographic parameters similar to those of glucose, they are not measured when [^{14}C]glucose is the substrate.
‖ Identity not definitely confirmed by cochromatography with authentic marker.
¶ Identity not definitely confirmed by cochromatography with authentic marker. However, it is chromatographically similar to lactic acid and fairly volatile. The volatility renders the values given above for the amounts of activity in lactic acid unreliable.

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Table 4. *Metabolism of* [^{14}C]*glucose and* [2-^{14}C]*acetate by rat brain, testis and heart muscle in the presence and absence of salicylate and 2:4-dinitrophenol*

Details were as given for Table 3, except that each flask contained 10·1 mg. dry wt. of brain, 4·4 mg. dry wt. of testis or 9·5 mg. dry wt. of heart muscle.

	Brain						Testis						Heart muscle					
Labelled substrate	[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate		
Uncoupling reagent	None	Sali-cylate	DNP	None	Sali-cylate	DNP	None	Sali-cylate	DNP	None	Sali-cylate	DNP	None	Sali-cylate	DNP	None	Sali-cylate	DNP
Sugar diphosphates*	0	0	0	0	0	0	0	0	0	0	0	0	0·8	0·5	2·6	0	0	0
Sugar monophosphates†	24	23	21	0	0	0	130	69	63	0	0	0	26	32	17	0	0	0
Phosphogluconic acid	0·2	0·2	0·5	0	0	0	6·3	11	14	0	0	0	0	0	0	0	0	0
Phosphoglyceric acid	1·0	0·1	0	0	0	0	0	9·7	0	0	0	0	0	0	0	0	0	0
Phosphoenolpyruvic acid	0	0	0	0	0	0	9·7	7·4	9·7	0	0	0	0·5	0·5	0·3	0	0	0
Uridine diphosphoglucose	1·2	0·7	0	0	0	0	17	10	8·5	0	0	0	0	0	0	0	0	0
Inosine triphosphate	0·2	0	0·2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	0	0	0	0	0	0	15	0	34	0	0	0	15	25	13	0	0	0
Fructose‡	31	7·4	36	0	0	0	180	13	100	0	0	0	42	0	0	0	0	0
Aspartic acid	200	220	100	32	6·4	5·0	84	85	76	9·7	4·0	6·3	44	37	38	13	7·4	6·6
Glutamic acid	150	290	210	41	8·7	13	480	370	130	190	46	54	0	0	0	13	6·1	2·1
Alanine	210	190	390	4·2	0	0	470	250	290	0·6	7·4	9·7	26	85	81	0	0	0
γ-Aminobutyric acid	110	83	69	17	5·7	3·7	120	0	0	0	0	0	0	0	0	0	0	0
Glutamine	5·7	0	13	0·2	0	0	0	0	0	16	8·0	0	0	0	0	0	0	0
Cysteine	0·7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glycine + serine§	—	—	—	0	0	0	—	—	—	0	0	0	—	—	—	2·4	0	0·8
Phenylalanine + leucine‖	0	0	0	0	0	0	23	4·0	57	14	0	0·3	24	37	10	19	16	17
Valine‖	6·9	46	50	0	0	0	150	150	110	30	17	27	56	47	45	0	0	0
Lactic acid¶	37	29	58	7·9	2·7	5·9	73	110	61	56	17	48	35	15	40	8·2	11	8·2
Fumaric acid	5·7	6·7	0	0·5	1·0	1·7	0	2·8	0	1·7	0	0	0	0	0	0	0	0
Malic acid	19	28	6·7	6·2	3·5	5·9	13	19	17	17	6·8	22	1·8	8·9	5·3	3·2	0	0
Citric acid	58	150	72	9·7	3·0	3·0	140	110	55	63	41	44	8·9	0	0	0	0	0
Succinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6·6	7·4	8·9
Total in:																		
Sugar phosphates	25	23	22	0	0	0	150	97	87	0	0	0	27	33	20	0	0	0
Uridine diphosphoglucose	1·2	0·7	0	0	0	0	17	10	8·5	0	0	0	0	0	0	0	0	0
Nucleotides	0·2	0	0·2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	0	0	0	0	0	0	15	0	34	0	0	0	15	25	13	0	0	0
Amino acids	680	830	830	96	20	22	1300	860	650	270	81	97	150	210	170	48	30	26
Organic acids	120	220	140	24	10	17	220	240	130	140	64	120	45	24	45	18	18	17
Unidentified compounds	6·9	14	13	12	8·4	10	170	300	220	43	32	54	84	130	100	4·4	0	4·6
Soluble fraction	870	1100	1000	130	40	49	2100	1500	1200	450	180	260	360	420	350	70	48	48

* Contains diphosphates of fructose and glucose.
† Contains monophosphates of glucose, fructose, ribose and sedoheptulose.
‡ Possibly a contaminant in the [^{14}C]glucose used as substrate. Its appearance as a separate spot may depend on a variable separation from the residual glucose.
§ Not separated chromatographically. As these two amino acids have chromatographic parameters similar to those of glucose, they are not measured when [^{14}C]glucose is the substrate.
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(Table 2) was found to involve the amino and organic acid fractions, particularly aspartate, glutamate, alanine and citrate.

DISCUSSION

The results show that chopped preparations of rat tissues incorporate measurable amounts of radioactivity from [^{14}C]glucose and [2-^{14}C]acetate into soluble metabolic intermediates. Thus despite the necessarily large degree of damage undergone by the tissues during their removal from the animal and subsequent preparation, it was possible to show the presence of radioactive substances characteristic of several metabolic pathways. These included glycolysis, the hexose monophosphate oxidative sequence, the tricarboxylic acid cycle and transamination. The majority of radioactive intermediates found were concerned with the catabolism rather than the anabolism of the labelled substrates. This was especially true of the experiments with [^{14}C]glucose. However, ^{14}C from the labelled acetate was found to be incorporated by liver and kidney into phosphoenolyruvate, phosphoglycerate and sugar monophosphates, which indicated the presence of synthetic pathways.

Salicylate (5 mM) and 0·5 mM-DNP had little effect on the total incorporation of ^{14}C from the [^{14}C]glucose into the soluble intermediates of the tissue, but produced a substantial decrease in that from the labelled acetate. This effect was particularly prominent in liver and kidney, which showed the largest incorporation of radioactivity from the acetate compared with that from glucose. The entry of the acetate carbon atoms into the metabolic sequences of the tissues therefore appears to be more dependent on the supply of adenosine triphosphate (ATP) from oxidative-phosphorylation processes than is the entry of the glucose carbon atoms. Unlabelled glucose was present in all the incubation media in addition to the radioactive substrates: the ATP produced during glycolysis, and from other reactions unaffected by salicylate and DNP, appeared to be capable of maintaining the continued entry of glucose but not that of acetate.

The greatly reduced total incorporation of ^{14}C from the [2-^{14}C]acetate in the presence of salicylate and DNP was reflected in the decreased amounts of the isotope found in all fractions of the soluble intermediates. Radioactivity when present was virtually restricted to the amino and organic acids and the participation of the acetate carbon atoms in synthetic reactions in the liver and kidney was negligible. The uncoupling reagents therefore not only decreased the initial uptake of the labelled acetate but also inhibited the conversion of its carbon atoms into substances requiring ATP for their synthesis. These results are compatible with the observation (Smith, 1959) that the incorporation of radioactivity into the liver glycogen of the intact rat given an intraperitoneal injection of [2-^{14}C]acetate is inhibited by the concurrent administration of salicylate.

The distribution of the isotope from the [^{14}C]-glucose among the soluble intermediates indicated that the main catabolic pathways of glycolysis and the tricarboxylic acid cycle were not inhibited by the uncoupling agents. Only very small amounts of isotope were incorporated into phosphogluconic acid and sedoheptulose phosphate in all the experiments, and no conclusions could be drawn about the effects of salicylate and DNP on the hexose monophosphate oxidative pathway. The incorporation of ^{14}C into the amino acids was not affected by the uncoupling reagents, showing that transamination reactions were not inhibited. The only evidence relating to the effects of salicylate and DNP on synthetic pathways was the reduced incorporation of ^{14}C into uridine diphosphoglucose and into the small amounts of nucleotides formed by liver and kidney. The results with the maltose fraction in the five tissues were extremely variable, only the kidney showing a decreased amount of ^{14}C in this fraction in the presence of the uncoupling reagents.

No qualitative differences between the effects of 5 mM-salicylate and 0·5 mM-DNP were detected during the experiments. The general effects of the two substances observed in the present work would appear to be explicable in terms of their uncoupling action on oxidative-phosphorylation reactions in that synthetic reactions involving ATP were inhibited and catabolic reactions not affected.

SUMMARY

1. The effects of 5 mM-salicylate and 0·5 mM-2:4-dinitrophenol on the incorporation of ^{14}C from [^{14}C]glucose and [2-^{14}C]acetate into the soluble intermediates of chopped rat tissues has been studied.

2. In the absence of the uncoupling reagents, the tissue preparations incorporated the isotope into substances known to be involved in glycolysis, the hexose monophosphate oxidative pathway, the tricarboxylic acid cycle, transamination and synthetic reactions.

3. Salicylate and dinitrophenol considerably reduced the total incorporation of ^{14}C from the labelled acetate, and the subsequent distribution of the isotope was restricted to the amino and organic acid fractions of the soluble intermediates.

4. The total incorporation and distribution of the ^{14}C from the [^{14}C]glucose were not materially altered in the presence of salicylate and dinitrophenol.

5. Some implications of these results are discussed.

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REFERENCES

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.
Brody, T. M. (1956). *J. Pharmacol.* **117**, 39.
Chain, E. B. (1959). *Brit. med. J.* **2**, 709.
Fuller, R. C. (1956). *Science*, **124**, 1253.
Hastings, A. B., Teng, C., Nesbett, F. B. & Sinex, F. M. (1952). *J. biol. Chem.* **194**, 69.
Jeffrey, S. W. & Smith, M. J. H. (1959). *Biochem. J.* **72**, 462.
Katz, J. & Chaikoff, I. L. (1955). *Biochim. biophys. Acta*, **18**, 87.
McIlwaine, H. & Buddle, H. L. (1953). *Biochem. J.* **53**, 412.
Manchester, K. L., Randle, P. J. & Smith, G. H. (1958). *Brit. med. J.* **1**, 1028.
Moses, V. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 507. Ed. by Smith, I. London: Heinemann (Medical Books) Ltd.
Packer, L., Austen, F. K. & Knoblock, E. C. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 239.
Penniall, R., Kalnitsky, G. & Routh, J. I. (1956). *Arch. Biochem. Biophys.* **64**, 390.
Randle, P. J. & Smith, G. H. (1958). *Biochem. J.* **70**, 490.
Schmidt, G. (1955). *Methods in Enzymology*, vol. 2, p. 523. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Smith, M. J. H. (1955). *Biochem. J.* **59**, 52.
Smith, M. J. H. (1959). *J. biol. Chem.* **234**, 144.
Smith, M. J. H. & Jeffrey, S. W. (1956). *Biochem. J.* **64**, 589.
Smith, M. J. H. & Moses, V. (1960). *Biochem. J.* **74**, 17P.

Biochem. J. (1960) **76**, 585

Uncoupling Reagents and Metabolism

2. EFFECTS OF 2:4-DINITROPHENOL AND SALICYLATE ON GLUCOSE METABOLISM IN BAKER'S YEAST

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Studies by Smith & Moses (1960) have shown that most, if not all, of the effects of salicylate and 2:4-dinitrophenol on intermediary metabolism in several animal tissues supplied with labelled glucose or acetate could be explained on the known abilities of these compounds to uncouple oxidative phosphorylation.

The present paper is concerned with similar experiments with baker's yeast. Studies were made of the kinetics of incorporation of the label into different metabolites, samples of the reaction mixtures being taken for analysis at time intervals ranging from a few seconds to several hours after the addition of the labelled substrate. A preliminary report of this work has already been published (Moses & Smith, 1960).

EXPERIMENTAL

Block yeast (3 g.) (The Distillers Co. Ltd.) was suspended in water and centrifuged at 1600 *g* for 5 min. The volume of the wet-packed cells was 3·8 ml. Half the cells (starved cells) were suspended in 25 ml. of tap water and the other half ('growing' cells) in 25 ml. of the following medium (g./l.): glucose, 10; NH_4Cl, 2; KH_2PO_4, 5·44; $MgSO_4,7H_2O$, 0·1; NaCl, 0·1, dissolved in London tap water [containing approximately (p.p.m.): Ca, 100; Na, 20–25; K, 5; Mg, 5 (all as bicarbonates); free Cl_2, less than 0·05] and adjusted to pH 5 with KOH. Each suspension was shaken at 25° for 3 hr., the cells were centrifuged, washed twice with 2 mM-KH_2PO_4, adjusted to pH 5 with KOH and resuspended in 19 ml. of this solution, which resulted in a cell concentration of 100 μl. of wet-packed cells/ml. When required, the final suspension also contained 2:4-dinitrophenol (DNP) or sodium salicylate at the appropriate concentration.

For the study of kinetics, 15 ml. of cell suspension was placed in a 50 ml. round-bottom flask equipped with a standard joint into which was fitted a Kipps automatic tilt pipette [H. J. Elliott Ltd. (E-Mil), Pontypridd, Glam.] calibrated to deliver 1 ml. The flask was shaken in air at 25° and the reaction was started, after removal of the tilt pipette, by the addition of 150 μl. of a solution of [^{14}C]-glucose (37·5 μc; 1·5 μmole; final glucose concn. 0·1 mM). [^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks.

The tilt pipette was replaced immediately and samples (1 ml.) of the suspension were run directly into 4 ml. of ethanol at room temperature at the following times after the addition of the labelled substrate: 5, 10, 15, 30, 45, 60,

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90, 120 and 180 sec.; 10, 30, 60 and 180 min. There was some unavoidable variation of the exact times at which the first three samples were taken, and these are recorded below in the Results section.

In experiments in which three samples only were required, 1 ml. of cell suspension was placed in each of three 25 ml. Erlenmeyer flasks, and the reaction initiated by the addition of 10 μl. of a solution of [^{14}C]glucose (2·5 μc; 0·1 μmole; final glucose concn., 0·1 mM). The flasks were shaken at 25° for 3, 30 and 180 min., and the reaction was terminated by the addition to the flask of 4 ml. of ethanol.

The aqueous ethanolic suspension of the cells was heated to boiling and the cells were separated by centrifuging at 1600 *g* for 5 min. The supernatant was removed and the cell residues were extracted again with 2 ml. of boiling aqueous 20% (v/v) ethanol. The extraction of the cells was repeated with 2 ml. of boiling water, the cell residues were discarded and the supernatants were pooled and concentrated to 0·6 ml. under reduced pressure at 40°. The radioactive substances in each extract were separated by paper chromatography, two chromatograms being run for each extract, one to detect all the substances in the extract and another to obtain a better separation of the phosphate esters. The techniques for chromatography, radioautography, spot identification and assay of radioactivity in each spot were the same as those described by Smith & Moses (1960).

RESULTS

Glucose metabolism in the absence of uncoupling reagents

The results of the incubation with [^{14}C]glucose of a suspension of yeast cells previously incubated for 3 hr. in a growth medium are shown in Table 1. The amount of radioactivity in each substance is expressed as a percentage of the sum of the radioactivity appearing in all the compounds listed; residual [^{14}C]glucose remaining in the reaction mixture, as well as that metabolized to $^{14}CO_2$, proteins, polysaccharides etc., has been excluded from the calculations.

Evidence was obtained for the occurrence in the yeast cells of several well-known metabolic pathways. The presence of radioactivity in glucose monophosphate, fructose monophosphate, fructose diphosphate, glucose diphosphate, phosphoglyceric acid and phosphoenolpyruvic acid supports the presence of the glycolytic sequence, and radioactivity in ribose monophosphate, sedoheptulose monophosphate and phosphogluconic acid indicates the operation of the pentose phosphate cycle. The presence of labelled phosphoglycollic acid and sedoheptulose diphosphate suggests the involvement of transketolase.

A number of radioactive citric acid-cycle intermediates were present (citric acid, fumaric acid, malic acid and succinic acid) as well as amino acids derived directly from citric acid-cycle intermediates by transamination (aspartic acid and glutamic acid). ^{14}C in alanine was probably derived by transamination from pyruvate. The presence of ^{14}C in glucose monophosphate, uridine diphosphoglucose and trehalose monophosphate supports the synthetic pathway for trehalose biosynthesis proposed by Leloir & Cabib (1953). Indeed trehalose was the only active disaccharide found, and at a late stage in the incubation more activity was found in this substance than in any other.

At the shortest incubation periods, most of the ^{14}C present in the soluble extract was confined to initial products of glucose metabolism (sugar phosphates) and relatively little radioactivity was found in substances such as amino acids and disaccharide; unchanged [^{14}C]glucose in the suspension gradually decreased in amount during the course of the experiment and had virtually disappeared after 3 hr. As the experiment progressed, lesser percentages of the radioactivity were found in the sugar phosphates, uridine diphosphoglucose and some organic acids, and ever greater percentages of the total ^{14}C appeared in the amino acids and trehalose. The radioactivity in the nucleotides remained low at all times.

Glucose metabolism in the presence of 2:4-dinitrophenol

A preliminary manometric experiment, to investigate the rate of endogenous oxygen uptake and the percentage of added glucose which was completely oxidized by the yeast, showed that at pH 5, 0·1 mM-DNP produced the greatest stimulation of the endogenous respiration and the greatest extent of complete oxidation of added glucose. This concentration of DNP was therefore used to study the effects of this substance on the metabolism of added [^{14}C]glucose by yeast previously suspended in growth medium for 3 hr.

Compared with results from the control experiment, the presence of DNP resulted in a decrease in the total ^{14}C found in the soluble extract of the cells, though the percentage of the total ^{14}C in the extract which was present in the sugar phosphates (particularly the hexose diphosphates, phosphoglyceric acid and phosphoenolpyruvic acid) remained relatively high for a longer period. The percentage of ^{14}C found in uridine diphosphoglucose, trehalose phosphate and free trehalose was much reduced, and no ^{14}C was found in trehalose phosphate until 30 sec., compared with 9 sec. for the control experiment. The percentages of ^{14}C in the various organic acids showed little variation from the control, but higher percentages were found in the amino acids, particularly in alanine, in which a high proportion of radioactivity was found throughout the experiment. Glutamic acid and aspartic acid contained a lower percentage of the total ^{14}C after the shorter incubation periods, and much higher percentages towards the end of the experiment (Table 2).

Table 1. *Kinetic study of* [^{14}C]*glucose metabolism by yeast*

Wet-packed cells (1·5 ml.), previously incubated in growth medium for 3 hr., were suspended in 15 ml. of 2 mM-KH_2PO_4 buffer, pH 5. [^{14}C]Glucose (37·5 μc, 1·5 μmole) was added and the suspension shaken at 25°. Samples (1 ml.) were removed as indicated below, the cells were killed and extracted with ethanol and the extracts chromatographed. The ^{14}C in each compound on the chromatogram was counted. The values given are expressed as the percentage of the total ^{14}C incorporated into all the soluble substances from labelled glucose, which is present in each individual substance. The ^{14}C remaining in the residual glucose was excluded from all calculations.

Incubation period (sec.)	9	12	18	30	45	60	90	120	180	600	1800	3600	10 800
Sugar diphosphates*	5·9	6·8	6·9	5·6	4·3	4·2	3·2	2·3	1·6	0·24	0·14	0·11	0·03
Sugar monophosphates†	37·6	42·2	42·5	39·4	33·5	26·1	22·0	16·7	12·0	2·4	0·60	1·3	1·2
Phosphoglyceric acid	1·2	1·5	2·3	2·9	3·7	4·3	4·9	4·9	5·4	2·0	0·90	0·48	0·38
Phosphogluconic acid	0·08	0·74	1·1	0·50	0·60	0·42	0·31	0·24	0·15	0·0	0·0	0·06	0·0
Trehalose phosphate	0·69	1·5	1·4	2·2	2·2	1·8	1·2	0·74	0·36	0·18	0·0	0·0	0·07
Phosphoenolpyruvic acid	1·1	0·56	0·85	1·1	1·2	1·4	2·1	2·6	2·6	0·69	0·39	0·21	0·15
Phosphoglycollic acid	0·29	0·33	0·30	0·30	0·17	0·20	0·09	0·12	0·15	0·0	0·08	0·10	0·10
Uridine diphosphoglucose	2·7	5·1	7·2	11·1	13·9	16·0	21·0	24·1	23·1	12·0	2·8	1·6	0·86
Uridine triphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·13	0·25	0·23	0·26	0·16
Guanosine diphosphate	0·0	0·0	0·0	0·0	0·0	0·07	0·0	0·13	0·21	0·28	0·61	0·32	0·26
Unidentified nucleoside monophosphate	0·0	0·98	0·0	0·0	0·0	0·21	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Unidentified nucleoside diphosphate	0·0	0·0	0·0	0·0	0·0	0·15	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Trehalose	3·1	3·4	5·8	10·3	14·3	17·4	20·5	29·0	29·7	43·4	53·7	58·8	62·2
Fructose	8·7	7·0	5·6	6·1	5·4	3·9	3·2	2·5	2·9	0·81	0·0	1·0	0·0
Cysteine	0·0	0·76	0·79	0·95	0·36	0·35	0·20	0·0	0·20	0·0	0·0	0·0	0·0
Aspartic acid	6·7	4·7	4·2	3·3	3·1	2·9	2·8	2·6	2·0	2·3	3·8	4·7	3·3
Glutamic acid	8·6	2·7	2·7	1·6	0·61	1·6	0·62	0·38	1·8	5·2	9·1	13·3	18·0
Alanine	0·0	2·6	2·9	4·6	5·4	6·5	6·0	4·6	6·1	20·2	19·7	10·8	7·5
Tyrosine	0·0	0·84	0·69	0·0	0·53	0·0	0·35	0·0	0·0	0·28	0·0	0·26	0·0
Glutamine	2·6	1·7	1·7	0·0	0·0	0·68	1·0	0·0	0·37	0·53	0·61	0·78	1·2
Phenylalanine + leucine	2·3	3·2	2·4	1·9	1·8	2·1	1·2	0·86	1·0	0·51	0·0	0·44	0·0
Valine	7·9	6·3	5·3	3·1	2·9	3·0	1·9	1·8	2·1	1·6	1·4	1·5	2·2
Citric acid	0·75	0·0	0·30	0·35	0·30	0·29	0·33	0·35	0·37	0·22	0·20	0·28	0·31
Fumaric acid	0·0	0·0	0·0	0·0	0·0	0·05	0·05	0·13	0·13	0·57	0·31	0·10	0·0
Malic acid	0·0	0·0	0·0	0·0	0·0	0·28	0·30	0·19	0·44	2·2	1·6	0·51	0·32
Succinic acid	2·6	0·88	0·38	0·46	0·56	1·3	0·76	0·30	0·94	1·1	0·34	0·64	0·0
Glyceric acid	4·4	5·6	3·9	3·6	5·0	3·0	5·3	5·3	6·1	2·9	2·7	1·7	1·5
Unidentified substances	2·6	0·64	0·78	0·55	0·22	1·7	0·11	0·09	0·23	0·15	0·52	0·80	0·34
Total in sugar phosphates	46·9	53·6	55·2	52·1	45·8	38·4	34·4	27·7	22·2	5·6	2·6	2·3	1·9
Total in uridine diphosphoglucose	2·7	5·1	7·2	11·1	13·9	16·0	21·0	24·1	23·1	12·0	2·8	1·6	0·86
Total in nucleotides	0·0	0·98	0·0	0·0	0·0	0·42	0·0	0·13	0·34	0·53	0·84	0·58	0·42
Total in free sugars	11·8	10·5	11·4	16·4	19·7	21·3	23·6	31·5	32·6	44·2	53·7	59·8	62·2
Total in amino acids	28·1	22·8	20·6	15·5	14·7	17·2	14·1	10·2	13·6	30·6	34·6	31·8	32·1
Total in organic acids	7·8	6·5	4·5	4·4	5·8	5·0	6·8	6·3	7·9	6·9	5·2	3·2	2·1
10^{-5} × Total activity (counts/min./100 μl. of cells)	0·34	0·62	0·71	1·03	1·19	1·51	1·65	1·86	2·25	2·12	1·97	2·08	1·89

* Contains diphosphates of glucose, fructose and sedoheptulose.

† Contains monophosphates of glucose, fructose, sedoheptulose and ribose.

Table 2. *Kinetic study of [^{14}C]glucose metabolism by yeast in the presence of 2:4-dinitrophenol*

Experimental conditions were the same as given for Table 1, except that the reaction mixture during incubation with [^{14}C]glucose contained 0·1 mM-2:4-dinitrophenol.

Incubation period (sec.)	6	12	15	30	45	60	90	120	180	600	1800	3600	10 800
Sugar diphosphates*	8·3	10·8	9·7	4·8	2·2	2·8	1·8	6·1	2·0	0·20	0·34	0·19	0·0
Sugar monophosphates†	31·4	42·9	40·8	32·5	26·5	26·3	22·6	17·5	16·7	1·9	1·3	0·74	0·94
Phosphoglyceric acid	7·4	14·0	13·8	13·2	12·5	16·9	14·0	13·2	8·5	0·78	0·12	0·22	0·27
Phosphogluconic acid	0·64	0·87	0·40	0·47	0·20	0·35	0·32	0·32	0·19	0·0	0·0	0·0	0·0
Trehalose phosphate	0·0	0·0	0·0	0·08	0·14	0·10	0·23	0·0	0·08	0·0	0·0	0·0	0·0
Phosphoenolpyruvic acid	3·3	1·1	1·8	4·6	4·0	3·3	3·8	3·8	2·6	0·47	0·11	0·01	0·05
Phosphoglycollic acid	0·0	0·0	0·0	0·0	0·0	0·10	0·0	0·25	0·07	0·13	0·0	0·12	0·14
Uridine diphosphoglucose	0·71	1·3	1·6	2·1	2·6	3·8	5·9	6·7	8·4	8·8	4·3	2·8	1·3
Uridine triphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·30	0·17	0·21	0·24
Guanosine diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·06	0·55	0·13	0·77
Unidentified nucleoside monophosphate	0·0	1·1	1·1	0·28	0·14	0·09	0·63	0·56	0·34	0·12	0·30	0·03	0·0
Unidentified nucleoside diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·13	0·0	0·26	0·0
Trehalose	1·1	0·69	4·2	3·0	2·7	2·4	1·4	1·9	2·6	3·2	4·0	4·3	5·8
Fructose	14·0	4·9	3·8	3·9	4·0	2·7	2·1	0·93	1·3	0·0	0·0	0·0	0·0
Cysteine	0·31	0·0	0·26	0·0	0·26	0·0	0·20	0·0	0·11	0·0	0·16	0·0	0·0
Aspartic acid	5·0	2·5	1·5	1·8	1·5	1·1	2·0	1·4	1·6	2·1	2·1	2·8	15·0
Glutamic acid	0·0	0·0	0·0	0·56	1·1	1·7	3·0	4·6	6·3	17·5	34·2	47·7	48·7
Alanine	12·3	13·5	13·9	24·8	31·2	30·6	29·6	33·5	38·9	55·8	42·3	29·8	15·3
Tyrosine	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·16	0·0	0·0	0·0	0·07	0·0
Glutamine	0·0	0·0	0·0	0·0	0·0	0·0	1·8	1·7	0·56	0·0	0·94	1·7	2·9
Phenylalanine + leucine	1·7	0·0	0·73	0·49	1·0	0·20	0·74	0·08	0·08	0·08	0·10	0·04	0·0
Valine	3·7	1·8	1·5	0·58	0·65	0·46	0·46	0·86	1·6	1·9	1·6	1·1	1·2
Citric acid	0·0	0·16	0·25	0·38	0·57	0·36	0·74	0·75	0·85	0·47	0·73	0·62	0·38
Fumaric acid	0·0	0·0	0·0	0·0	0·0	0·06	0·10	0·23	0·41	0·67	0·88	0·46	0·28
Malic acid	0·0	0·0	0·0	0·0	0·15	0·45	0·31	0·70	1·4	1·6	2·4	2·3	1·0
Succinic acid	1·1	0·0	0·53	0·67	0·54	0·63	0·15	0·90	0·96	1·0	0·79	0·88	1·0
Glyceric acid	6·1	4·4	4·3	5·4	7·9	5·1	6·8	2·6	2·4	0·32	0·28	0·10	0·17
Unidentified substances	2·9	0·0	0·0	0·59	0·0	0·55	1·6	1·4	2·2	2·5	2·4	3·2	4·6
Total in sugar phosphates	51·1	69·7	66·4	55·6	45·6	49·9	42·4	41·1	30·1	3·5	1·9	1·3	1·4
Total in uridine diphosphoglucose	0·71	1·3	1·6	2·1	2·6	3·8	5·9	6·7	8·4	8·8	4·3	2·8	1·3
Total in nucleotides	0·0	1·1	1·1	0·28	0·14	0·09	0·63	0·56	0·34	0·51	1·0	0·63	1·0
Total in free sugars	15·1	5·6	8·0	6·9	6·7	5·1	3·5	2·8	3·9	3·2	4·0	4·3	5·8
Total in amino acids	23·0	17·7	17·9	28·2	35·7	34·1	37·8	42·3	29·1	77·4	81·1	83·5	82·9
Total in organic acids	7·2	4·6	5·1	6·5	9·1	6·6	8·1	5·2	6·0	4·1	5·0	4·4	2·9
10^{-5} × Total activity (counts/min./100 μl. of cells)	0·19	0·24	0·26	0·53	0·72	0·74	0·75	0·93	0·98	0·93	1·10	1·14	0·88

* Contains diphosphates of glucose, fructose and sedoheptulose.

† Contains monophosphates of glucose, fructose, sedoheptulose and ribose.

Table 3. *Kinetic study of* [^{14}C]*glucose metabolism by yeast in the presence of sodium salicylate*

Experimental conditions were the same as given for Table 1, except that the reaction mixture during incubation with [^{14}C]glucose contained 8 mM-sodium salicylate.

Incubation period (sec.)	6	11	15	30	45	60	90	120	180	600	1800	3600	10 800
Sugar diphosphates*	0·94	1·6	2·1	2·7	2·2	2·1	2·0	1·2	1·0	0·18	0·0	0·0	0·0
Sugar monophosphates†	17·2	22·2	27·6	30·7	29·5	27·4	25·3	19·7	9·8	2·1	1·0	1·5	1·6
Phosphoglyceric acid	1·6	1·1	1·7	4·3	5·8	4·1	3·7	3·4	2·4	0·84	0·20	0·16	0·24
Phosphogluconic acid	0·32	0·77	0·48	0·47	0·42	0·27	0·46	0·05	0·22	0·04	0·06	0·09	0·08
Trehalose phosphate	0·0	0·0	0·0	0·28	0·24	0·50	0·39	0·45	0·18	0·22	0·21	0·0	0·0
Phosphoenolpyruvic acid	0·59	0·64	0·86	0·90	1·0	0·90	0·92	0·82	0·93	0·21	0·09	0·10	0·16
Phosphoglycollic acid	0·0	1·3	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Uridine diphosphoglucose	0·64	1·4	2·1	3·5	4·8	6·4	9·7	10·8	13·9	10·1	4·5	3·6	1·5
Uridine triphosphate	0·0	0·0	0·96	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·05	0·27	0·25
Guanosine diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·16	0·24	0·37	0·72	0·32
Unidentified nucleoside monophosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·32	0·0	0·30
Unidentified nucleoside diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·13	0·15	0·16	0·17
Trehalose	5·8	5·7	3·4	4·7	3·7	3·2	7·1	7·6	8·8	9·8	11·7	12·9	16·2
Fructose	13·3	11·6	13·9	11·8	13·0	11·9	11·8	11·6	6·2	1·9	0·60	0·0	0·0
Cysteine	1·5	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Aspartic acid	13·7	10·7	9·6	7·9	5·6	4·0	4·2	4·3	3·3	2·6	3·1	7·9	9·2
Glutamic acid	6·6	5·7	4·1	3·6	2·4	3·0	2·2	2·7	6·9	17·8	34·3	21·5	40·7
Alanine	7·4	4·9	6·2	7·2	9·7	12·1	8·9	14·7	21·9	39·4	29·5	33·8	16·9
Tyrosine	0·0	1·6	1·4	0·0	0·50	0·41	0·91	0·53	0·75	0·23	0·0	0·0	0·0
Glutamine	0·0	2·8	0·0	0·0	0·0	0·0	0·78	0·0	0·0	0·0	0·59	1·3	2·7
Phenylalanine + leucine	4·6	3·9	4·3	3·2	2·7	2·5	2·1	3·1	1·6	0·64	0·93	1·4	0·78
Glutathione	0·0	2·3	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Valine	7·4	6·4	5·9	4·1	3·0	3·6	4·1	4·1	3·5	1·7	1·5	2·3	1·7
Citric acid	0·0	0·0	0·41	0·34	0·81	1·4	2·8	3·1	5·3	2·6	0·83	0·78	0·50
Fumaric acid	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·26	0·51	0·60	0·90	0·23
Malic acid	0·0	0·0	0·0	0·18	0·23	0·13	0·34	0·40	0·97	2·0	2·8	3·2	1·1
Succinic acid	0·68	0·24	0·30	0·0	0·20	0·90	1·1	0·30	0·52	2·3	2·3	2·1	1·4
Glyceric acid	9·7	9·4	8·1	10·7	12·1	11·8	10·3	11·0	10·7	4·3	1·6	1·6	1·8
Unidentified substances	8·0	5·6	6·7	3·6	2·0	2·0	0·90	0·41	0·67	0·12	2·7	3·3	1·9
Total in sugar phosphates	20·6	27·8	32·6	39·1	39·3	35·3	32·7	25·6	14·5	3·7	1·6	1·8	2·0
Total in uridine diphosphoglucose	0·64	1·4	2·1	3·5	4·8	6·4	9·7	10·8	13·9	10·1	4·5	3·6	1·5
Total in nucleotides	0·0	0·0	0·96	0·0	0·0	0·0	0·0	0·0	0·16	0·37	0·89	1·2	1·0
Total in free sugars	19·1	17·3	17·3	16·5	16·7	15·1	18·9	19·2	15·0	11·7	12·3	12·9	16·2
Total in amino acids	41·2	38·3	31·5	26·0	23·9	27·2	23·1	29·5	37·9	62·4	69·9	68·5	72·2
Total in organic acids	10·4	9·6	8·9	11·2	13·3	14·2	14·6	14·9	17·8	11·6	8·2	8·5	5·1
10^{-5} × Total activity (counts/min./100 μl. of cells)	0·25	0·28	0·39	0·55	0·74	0·82	0·82	1·02	1·12	1·22	1·50	1·16	1·29

* Contains diphosphates of glucose, fructose and sedoheptulose.

† Contains monophosphates of glucose, fructose, sedoheptulose and ribose.

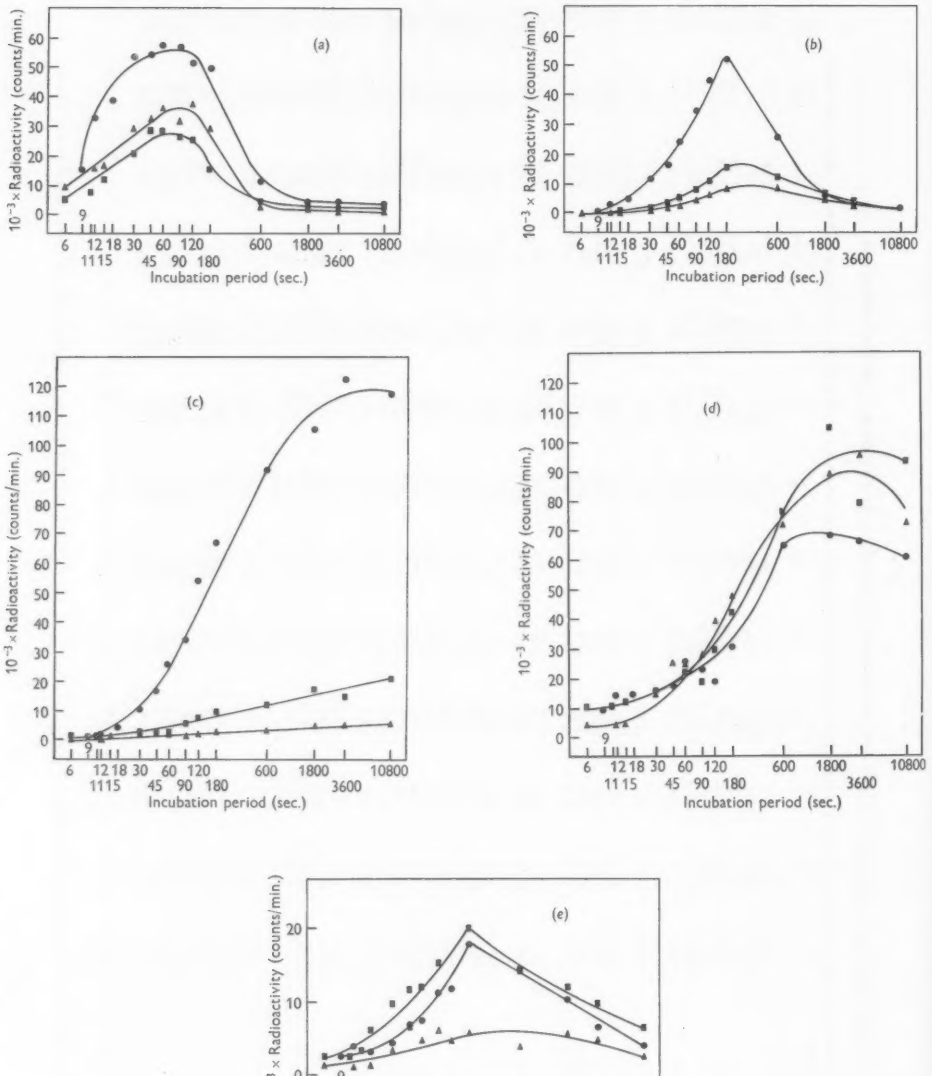

Fig. 1. Incorporation of ^{14}C from [^{14}C]glucose into various groups of compounds by baker's yeast. Experimental details were as given for Tables 1–3. Results are plotted as counts/min./100 μl. of wet-packed cells of the ^{14}C incorporated into each group of compounds. (*a*) Sugar phosphates, (*b*) uridine diphosphoglucose, (*c*) trehalose, (*d*) amino acids, (*e*) organic acids. ●, Control with no uncoupling reagent; ▲, with 0·1 mM-DNP; ■, with 8 mM-salicylate.

If the values recorded in Tables 1 and 2 are expressed as the actual quantities of ^{14}C present in each compound, rather than as a percentage of the total soluble ^{14}C, it was found that, compared with the controls, cells incubated in the presence of DNP contained less radioactivity in the sugar phosphates, uridine diphosphoglucose and trehalose, and more in the amino and organic acids. Little change was observed in the nucleotide fraction (Fig. 1).

Glucose metabolism in the presence of sodium salicylate

A manometric experiment similar to that mentioned above showed that, at pH 5, 10 mM-salicylate produced the maximum stimulation of the endogenous respiration of the yeast and caused the greatest oxidation of added glucose. However, as the rates of respiration, both with and without added glucose, fell very rapidly at concentrations of salicylate higher than 10 mM, a concentration of 8 mM was used to avoid the possibility of inhibiting respiratory processes.

Table 3 shows the results of incubating yeast cells with [^{14}C]glucose in the presence of 8 mM-sodium salicylate. Expressed on a percentage basis, sodium salicylate had a similar effect to DNP on the distribution of ^{14}C among the products of glucose metabolism, with the following minor differences: compared with the controls incubated in the absence of uncoupling reagents, salicylate-

Table 4. *Effect of high concentrations of 2:4-dinitrophenol and sodium salicylate on [^{14}C]glucose metabolism in yeast*

Each flask contained 0·1 ml. of wet-packed cells, previously incubated in growth medium for 3 hr., suspended in 1 ml. of 2 mM-KH_2PO_4 buffer, pH 5. [^{14}C]Glucose (2·5 μC, 0·1 μmole) was added and the suspension incubated at 25°. When present, the concn. of 2:4-dinitrophenol was 1 mM, and that of sodium salicylate, 50 mM. The cells were killed after the appropriate incubation period by the addition of 4 ml. of ethanol. Other details were as given for Table 1.

Uncoupling agent	None			2:4-Dinitrophenol			Sodium salicylate		
Incubation period (sec.) ...	180	1800	10 800	180	1800	10 800	180	1800	10 800
Sugar diphosphates*	0·65	0·18	0·34	0·70	0·0	0·0	0·72	0·0	0·0
Sugar monophosphates†	15·3	1·7	1·9	4·4	2·4	0·90	18·0	0·80	0·79
Phosphoglyceric acid	7·0	2·3	0·47	7·4	0·0	0·24	5·4	0·56	0·38
Phosphogluconic acid	0·46	0·03	0·51	0·27	0·15	0·05	0·43	0·17	0·0
Trehalose phosphate	0·56	0·08	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Phosphoenolpyruvic acid	3·6	4·2	0·12	4·8	0·51	0·98	4·4	1·9	0·74
Uridine diphosphoglucose	3·3	0·44	2·0	0·89	2·0	0·13	0·82	3·2	1·5
Uridine triphosphate	0·10	0·15	0·10	0·0	0·0	0·03	0·0	0·24	0·44
Guanosine diphosphate	0·21	0·24	0·87	0·0	0·0	0·0	0·0	0·15	0·29
Adenosine triphosphate	0·0	0·14	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Unidentified nucleoside monophosphate	0·44	0·10	0·45	0·0	0·0	0·0	0·0	0·96	0·56
Trehalose	31·7	49·3	51·3	1·9	1·4	0·86	8·5	8·5	11·9
Fructose	2·0	0·0	0·0	0·0	0·0	1·4	8·0	0·0	2·1
Aspartic acid	1·9	3·8	3·3	4·0	3·7	2·3	4·4	3·2	3·3
Glutamic acid	1·2	10·1	17·1	13·7	32·5	54·3	2·2	18·0	30·1
Alanine	3·3	14·3	10·1	28·6	39·1	20·7	12·9	28·3	23·9
Tyrosine	0·0	0·42	1·0	3·5	1·9	1·1	0·0	0·0	0·0
Glutamine	0·72	0·21	1·3	2·1	0·79	1·7	3·5	1·2	1·2
Phenylalanine + leucine	1·3	1·8	1·2	3·2	1·6	1·2	1·6	3·4	1·2
Valine	1·2	0·94	2·2	4·0	3·0	2·7	4·8	3·3	4·1
Citric acid	8·7	2·7	0·17	3·2	0·35	0·69	3·9	6·5	0·54
Fumaric acid	1·1	0·40	0·06	0·28	0·18	0·13	0·0	0·66	0·29
Malic acid	0·84	0·59	0·27	1·3	1·7	0·95	0·93	1·6	0·94
Succinic acid	6·3	2·2	0·34	8·9	0·0	1·4	8·9	2·7	1·6
Glyceric acid	4·2	1·1	2·0	3·0	4·4	1·7	5·0	4·8	4·0
Unidentified substances	4·2	2·6	3·1	4·0	4·3	6·5	5·5	9·8	10·1
Total in sugar phosphates	27·5	8·4	3·3	17·6	3·1	2·1	29·0	3·5	1·9
Total in uridine diphosphoglucose	3·3	0·44	2·0	0·89	2·0	0·13	0·82	3·2	1·5
Total in nucleotides	0·75	0·63	1·4	0·0	0·0	0·03	0·0	1·3	1·3
Total in free sugars	33·7	49·3	51·3	1·9	1·4	2·3	16·5	8·5	14·0
Total in amino acids	9·5	31·6	36·1	59·0	82·6	84·0	29·4	57·3	63·8
Total in organic acids	21·1	7·1	2·8	16·7	6·7	4·9	18·8	16·3	7·3
10^{-5} × Total activity (counts/min./100 μl. of cells)	2·52	2·25	1·69	0·76	1·04	1·22	0·81	0·83	1·02

* Contains diphosphates of glucose, fructose and sedoheptulose.
† Contains monophosphates of glucose, fructose, sedoheptulose and ribose.

treated cells showed a smaller percentage incorporation of ^{14}C into the sugar phosphates (particularly the monophosphates, diphosphates, phosphogluconic acid and phosphoenolpyruvic acid) and a greater incorporation into glyceric acid, and into aspartic acid, and alanine throughout the course of the experiment. However, the percentage incorporation into alanine was not as high as in the presence of DNP.

On the basis of the absolute radioactivity in each compound, the results with salicylate compared with those from controls are similar to those with DNP except that with salicylate more activity was found in the organic acids (Fig. 1). When the absolute activities obtained in the presence of salicylate are compared with those found in the presence of DNP, salicylate-treated cells had more activity in uridine diphosphoglucose, trehalose and the organic acids, and about the same in the nucleotides and in the soluble extract as a whole. However, less radioactivity was found in the sugar phosphates (Fig. 1).

Table 5. *Effect of 2:4-dinitrophenol and sodium salicylate on* [^{14}C]*glucose metabolism in starved yeast*

Experimental conditions were as given in Table 4, except that the yeast cells were previously starved by incubation for 3 hr. at 25° in 2 mM-KH_2PO_4 buffer, pH 5. The concentrations of 2:4-dinitrophenol and sodium salicylate in the incubation medium were 0·1 mM and 8 mM respectively.

Uncoupling agent	None			2:4-Dinitrophenol			Sodium salicylate		
Incubation period (sec.) ...	180	1800	10 800	180	1800	10 800	180	1800	10 800
Sugar diphosphates*	1·6	0·08	0·0	0·95	0·06	0·0	0·73	0·04	0·0
Sugar monophosphates†	15·4	2·2	2·7	14·7	4·7	0·80	7·6	1·5	1·6
Phosphoglyceric acid	6·4	2·3	0·68	3·8	1·3	0·0	2·7	0·59	0·22
Phosphogluconic acid	0·0	0·0	0·0	0·30	0·0	0·0	0·22	0·0	0·08
Trehalose phosphate	0·40	0·0	0·0	0·13	0·0	0·0	0·47	0·0	0·0
Phosphoenolpyruvic acid	1·6	0·58	0·16	3·0	0·24	0·07	0·81	0·15	0·04
Phosphoglycollic acid	0·25	0·11	0·20	0·0	0·0	0·0	0·0	0·0	0·0
Uridine diphosphoglucose	22·8	11·2	6·2	8·9	0·26	1·7	16·4	6·5	2·8
Uridine triphosphate	0·15	0·40	0·46	0·0	0·0	0·14	0·0	0·25	0·44
Guanosine diphosphate	0·32	1·0	1·4	0·13	0·37	0·56	0·23	0·48	0·24
Unidentified nucleoside monophosphate	0·0	0·0	0·0	0·27	0·0	0·0	0·0	0·29	0·0
Unidentified nucleoside diphosphate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·73
Trehalose	19·3	31·8	37·8	1·7	3·3	7·0	9·4	13·0	17·8
Fructose	3·4	0·0	0·0	2·8	0·15	0·0	7·3	1·4	1·3
Cysteine	0·0	0·0	0·0	0·04	0·0	0·0	0·0	0·0	0·0
Aspartic acid	2·8	5·4	5·5	2·0	8·6	11·6	4·1	5·4	7·2
Glutamic acid	1·8	16·5	23·2	10·9	37·0	50·7	8·1	31·1	38·3
Alanine	7·4	18·8	11·1	26·3	36·7	20·5	15·2	24·8	17·1
Tyrosine	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·34	0·25
Glutamine	1·1	0·84	1·0	1·4	0·30	0·97	0·0	1·1	1·1
Phenylalanine + leucine	1·6	1·0	1·1	0·93	0·72	0·75	1·5	1·3	1·1
Glutathione	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·12	0·19
Valine	2·0	1·3	1·3	0·92	0·12	0·33	2·9	1·7	2·2
Citric acid	0·40	1·2	2·4	1·1	1·0	1·2	3·6	1·7	2·0
Fumaric acid	0·0	0·0	0·0	0·30	0·37	0·08	0·17	0·27	0·09
Malic acid	0·0	0·27	0·37	0·0	0·0	0·42	0·76	1·4	0·48
Succinic acid	0·0	0·21	0·31	1·1	1·2	1·2	0·50	0·34	0·53
Glyceric acid	11·3	4·5	1·7	18·1	2·6	0·22	17·5	3·6	1·8
Unidentified substances	0·0	0·24	2·2	0·57	1·1	1·8	0·24	2·3	2·6
Total in sugar phosphates	25·7	5·3	3·7	22·9	6·3	0·87	12·5	2·3	1·9
Total in uridine diphosphoglucose	22·8	11·2	6·2	8·9	0·26	1·7	16·4	6·5	2·8
Total in nucleotides	0·47	1·4	1·9	0·40	0·37	0·70	0·23	1·0	1·4
Total in free sugars	22·7	31·8	37·8	4·5	3·5	7·0	16·7	14·4	19·3
Total in amino acids	16·6	43·8	43·4	42·4	83·3	84·9	31·7	66·3	67·9
Total in organic acids	11·7	6·2	4·8	20·6	5·2	3·1	22·5	7·4	4·8
10^{-5} × Total activity (counts/min/100 μl. of cells)	1·86	2·03	1·59	1·07	1·71	1·12	1·14	2·08	1·77

* Contains diphosphates of glucose, fructose and sedoheptulose.
† Contains monophosphates of glucose, fructose, sedoheptulose and ribose.

Effects of high concentration of uncoupling reagents on [^{14}C]glucose metabolism

Further experiments were performed to investigate the effects on labelled glucose metabolism of concentrations of DNP (1 mM) and salicylate (50 mM) that were known from preliminary manometric data to be inhibitory both to the endogenous respiration of the yeast and to the rate of oxygen uptake with glucose as substrate. Three incubation periods only were used: 3, 30 and 180 min.

The changes in percentage distribution in the presence of the higher concentrations of uncouplers did not differ from those produced by the lower concentrations: there was, however, a small decrease in the total amount of ^{14}C incorporated into the soluble extract (Table 4).

Effect of uncoupling agents on [^{14}C]glucose metabolism in starved cells

Starved cells were incubated with labelled glucose in the presence and absence of 0·1 mM-DNP or 8 mM-sodium salicylate for 3, 30 and 180 min. (Table 5). The experiments were performed at the same times as those reported in Tables 1–3 and used the same starting material (see Experimental section).

In the absence of uncoupling reagents, the main effects of starvation (cf. Table 1) were to reduce the total ^{14}C incorporated into the soluble extract, and to reduce the percentage radioactivity found in trehalose. However, a higher percentage of the radioactivity was found in uridine diphosphoglucose, the nucleotides and glyceric acid at the longer incubation periods, and in the amino acids, sugar monophosphates and phosphoglyceric acid during the whole incubation.

In the presence of the uncoupling reagents, compared with the appropriate experiments with unstarved cells (Tables 2 and 3), the effect of starvation was to increase the total soluble ^{14}C and to cause some alteration in the percentage distribution among the various amino acids.

DISCUSSION

Routes of glucose metabolism in baker's yeast

The present studies have confirmed the work of Aubert & Milhaud (1955), who found ^{14}C in a number of sugar phosphates, amino acids, organic acids, uridine diphosphate derivatives and trehalose when yeast was incubated with labelled glucose. Glucose is thus metabolized through glycolysis, the pentose phosphate cycle, the citric acid cycle and via uridine diphosphoglucose to trehalose phosphate and free trehalose. It was impossible to estimate in the present work either the relative importance of the glycolytic and hexose monophosphate-shunt mechanisms, or the significance of the citric acid cycle as an oxidative pathway rather than a synthetic mechanism for the production of amino acids. Beevers & Gibbs (1954), however, suggested that the pentose phosphate cycle may be the main route of glucose breakdown, and Krebs, Gurin & Eggleston (1952) believe that the principal function of the citric acid cycle is for synthesis and not for the oxidation of C_2 fragments to carbon dioxide.

The incorporation of ^{14}C into all the substances investigated can be explained by well-established biochemical pathways. However, the role in metabolism of free fructose and free glyceric acid, both of which contained appreciable amounts of radioactivity, is not clear. Chromatographic analysis of the glucose revealed traces of fructose. Alternatively it is possible that yeast forms and subsequently utilizes free fructose, or that fructose is formed from a fructose phosphate by the momentary action of a phosphatase during the killing procedure with ethanol. Although glyceric acid was not a contaminant in the [^{14}C]glucose, whether its presence indicates that it was a genuine metabolic intermediate, possibly on the pathway of glycine and serine synthesis, or whether it may also have been formed by hydrolysis of a phosphate precursor, is uncertain.

Effects of uncoupling reagents

Although Lutwak-Mann (1942) found little effect of salicylate on the respiration of yeast at a concentration less than 0·1 M, the present studies have demonstrated effects on the uptake and distribution of ^{14}C from [^{14}C]glucose by both 0·1 mM-DNP and 8 mM-sodium salicylate. In addition to a number of minor variations between experiments, the predominant effect of the uncoupling reagents for the longer incubation periods (30–180 min.) was to suppress the absolute incorporation of ^{14}C into all classes of compounds except the amino acids, into which the incorporation was increased: the inhibition of incorporation of ^{14}C was most marked with trehalose. At shorter incubation periods (30–90 sec.) the overall picture was similar, except that the most marked inhibition was into uridine diphosphoglucose, trehalose phosphate and trehalose, and the incorporation into amino acids was either decreased or slightly stimulated (Fig. 1). Thus the observable effects of uncoupling reagents depend on the time at which the observation is made. In the early stages the incorporation of ^{14}C into the intermediates of certain metabolic sequences was inhibited, whereas during the later stages the inhibition was more pronounced in relation to the end products of those reaction sequences.

A comparison with the effect of an uncoupler of oxidative phosphorylation in another organism is provided by the observation that in starved *Zygorrhynchus moelleri* the incorporation of ^{14}C from [^{14}C]glucose into all classes of compounds except amino and organic acids was inhibited by 1 mM-sodium azide, with the most prominent inhibition affecting the nucleotides and maltose (Moses, 1959).

Most of the effects of DNP and salicylate are explicable on the basis of both of these substances being primarily uncouplers of oxidative phosphorylation and hence blocking the supply of energy for synthetic purposes. The more important compounds requiring energy for synthesis are uridine diphosphoglucose, trehalose, polysaccharides, nucleotides and proteins, and if their synthesis is prevented by an insufficiency of adenosine triphosphate (ATP) it may be expected that the compounds immediately preceding the blocked steps would accumulate unless there were alternative metabolic routes available for them to escape. Of the compounds requiring energy for synthesis, only uridine diphosphoglucose and trehalose have been directly measured. In the absence of uncouplers, a large amount of radioactivity appeared in trehalose after 3 hr., whereas very little appeared in the presence of DNP and salicylate. Glucose and ribose phosphates might be expected to accumulate, but these compounds can be converted through glycolysis or the pentose phosphate cycle into pyruvate and may not accumulate for this reason. The relative accumulation of amino acids and organic acids in the presence of the uncouplers is probably due to the fact that the citric acid cycle is a pathway primarily for amino acid synthesis and not for oxidation (Krebs *et al.* 1952), and the mechanisms for organic acid and amino acid oxidation to carbon dioxide may not operate at a sufficient rate to reduce the accumulation of amino acids.

In the restricted view of cellular metabolism that these experiments provides, no specific effects of salicylate have been found distinct from those of DNP, except that salicylate tends to be rather weaker in its action even at the most effective concentration for each uncoupler. Both substances appear from these studies to act solely as uncouplers of oxidative phosphorylation, a conclusion which also applies to the studies with animal tissues (Smith & Moses, 1960).

SUMMARY

1. Studies have been made of the metabolism of [^{14}C]glucose by baker's yeast previously suspended for 3 hr. in growth medium. Samples of the reaction mixture were taken at intervals from about 5 sec. to 3 hr. ^{14}C was incorporated into a number of compounds, which suggests that glucose is metabolized through glycolysis, the pentose phosphate cycle, the citric acid cycle and via uridine diphosphoglucose to trehalose phosphate and free trehalose.

2. Similar experiments performed in the presence of 0·1 mM-2:4-dinitrophenol and 8 mM-sodium salicylate showed that the main effect of these uncouplers of oxidative phosphorylation was to suppress partially the incorporation of ^{14}C into all compounds except the amino acids; the inhibition was most marked with uridine diphosphoglucose and trehalose.

3. The principal result of increasing the concentration of the uncouplers to 1 mM-2:4-dinitrophenol and 50 mM-salicylate was to depress still further the total incorporation of ^{14}C, though the distributional pattern of the isotope in a number of substances was similar to that with the lower concentrations.

4. Starving the yeast cells before the addition of [^{14}C]glucose resulted in a fall of ^{14}C incorporated into trehalose, and a rise in all other fractions except the organic acids. In experiments with the uncoupling agents, starvation resulted in an increase in the total ^{14}C taken up into the soluble extract of the cells and some distributional variation among the amino acids.

5. The effects of 2:4-dinitrophenol and salicylate on [^{14}C]glucose metabolism are explicable on the basis of their uncoupling action. Little difference was observed between the action of 2:4-dinitrophenol and that of salicylate.

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REFERENCES

Aubert, J. P. & Milhaud, G. (1955). *C.R. Acad. Sci., Paris*, **240**, 1943.

Beevers, H. & Gibbs, M. (1954). *Nature, Lond.*, **173**, 640.

Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.

Leloir, L. F. & Cabib, E. (1953). *J. Amer. chem. Soc.* **75**, 5445.

Lutwak-Mann, C. (1942). *Biochem. J.* **36**, 706.

Moses, V. (1959). *J. gen. Microbiol.* **20**, 184.

Moses, V. & Smith, M. J. H. (1960). *Biochem. J.* **74**, 18P.

Smith, M. J. H. & Moses, V. (1960). *Biochem. J.* **76**, 579.

Studies in Detoxication

82. THE FATE OF BENZOIC ACID IN SOME DOMESTIC AND OTHER BIRDS*

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In most laboratory mammals benzoic acid is converted into hippuric acid and benzoyl glucuronide (see Williams, 1959), except for the cat, which seems to produce only hippuric acid (Robinson & Williams, 1958, and unpublished data). Jaffe (1877) showed that benzoic acid was converted in hens into ornithuric acid, and benzoyl glucuronide has also been found to be a metabolite of benzoic acid in hens (Baldwin, Robinson & Williams, 1959). It has been assumed that ornithuric acid formation is common to most birds, but, as far as we are aware, there is no evidence to support this. Schachter, Manis & Taggart (1955) have shown that pigeon-kidney slices and extracts form glycine conjugates, whereas kidney preparations from the chicken, duck, goose and turkey do not. They also found that chicken- and duck-kidney powder formed ornithine conjugates. These studies *in vitro* suggest that ducks as well as chickens form ornithuric acids whereas pigeons form hippuric acids. In the present paper we shall show, with the intact bird, that chickens, turkeys, geese and ducks excrete ornithuric acid after being dosed with benzoic acid, whereas the pigeon and the related wood pigeon and dove excrete hippuric acid. Neither hippuric acid nor ornithuric acid was found in the excreta of a parrot and a carrion crow dosed with benzoic acid. All the birds examined formed benzoyl glucuronide.

MATERIALS AND METHODS

Materials. [*carboxy*-^{14}C]Benzoic acid was obtained from The Radiochemical Centre, Amersham. L(+)-Ornithuric acid was prepared by benzoylation of L(+)-ornithine; it had m.p. 182°, $[\alpha]_D^{23}$ +11·9° (*c*, 1 in 0·1 N-NaOH) and light absorption in 0·1 N-HCl, $\lambda_{max.}$ 229 ($\epsilon_{max.}$ 23 250) and 270 mμ ($\epsilon_{max.}$ 2500). A sample prepared from the excreta of hens dosed with benzoic acid had m.p. 178° and $[\alpha]_D^{23}$ + 7·9° (*c*, 2 in 0·1 N-NaOH). N^5-Benzoyl-L-ornithine, m.p. 243–245° and $[\alpha]_D^{19}$ +8·3° (*c*, 0·5 in water) was prepared according to McGilvery & Cohen (1950). N^5-Benzyloxycarbonyl-L-ornithine (4 g.; Barrass & Elmore, 1957) was benzoylated with benzoyl chloride and dilute NaOH to give N^2-benzoyl-N^5-benzyloxycarbonyl-L-ornithine (6 g., crude). The latter was dissolved in methanol (100 ml.) and water (100 ml.), treated with palladium oxide (0·5 g.) and then hydrogenated at a pressure of 38 lb./in.^{2} for 2 hr. The spongy palladium was filtered off and the solution evaporated. The N^2-benzoyl-L-ornithine (1·7 g.) was recrystallized from ethanol–water and had m.p. 239° and $[\alpha]_D^{19}$ +4·8° (*c*, 0·9 in water) (Found: N, 11·8. Calc. for $C_{12}H_{16}O_3N_2$: N, 11·85%). Karrer & Ehrenstein (1926) give m.p. 224–226° and $[\alpha]_D^{20}$ +8° in water for N^2-benzoyl-L-ornithine prepared by the action of $Ba(OH)_2$ solution on L-ornithuric acid.

Birds. Brown Leghorn laying pullets (6 months old) were obtained from the A.R.C. Poultry Research Centre, Edinburgh. They were maintained on 100 g. of Superbra Intensive Battery Mash (Poultry Association of Great Britain)/day and allowed ground oyster-shell grit and water without restriction. Pigeons, Muscovy ducks, a goose and a carrion crow were bought on the open market. The two turkeys, Broad-breasted Bronze hens, were gifts. The other birds were obtained on loan from the Zoological Society of London.

Benzoic acid suspended in water containing a little bile salts was administered to the hens, ducks and turkeys by crop tube. With other birds the benzoic acid was enclosed in a small gelatin capsule or compressed into a small tablet and given by mouth.

Collection of excreta. Food, but not water, was withdrawn from the birds on the evening before benzoic acid administration, to reduce the amount of faeces excreted in the first 24 hr. after dosing. With the hens, the urine was collected in a flask containing 2 ml. of saturated aqueous mercuric chloride. The funnel of the metabolism cage was scraped and rinsed with water and, where necessary in the experiments with ^{14}C, the birds' feet were also rinsed with water. With pigeons and the smaller birds, the excreta were collected on a glass plate placed under the wide-mesh bottom of the cage. The excreta were scraped from the plate and cage, which were then rinsed with ethanol and dilute ammonia solution. A thin paste of excreta was thus obtained. For paper chromatography the paste was centrifuged and the supernatant was used. The urine of the turkeys and goose was collected as for hens, but no mercuric chloride was used.

Measurement of radioactivity. All measurements were carried out on solid or liquid samples of 'infinite thickness' in an end-window counter. The specific activities were determined by comparison with a stable reference polymer (The Radiochemical Centre) and sufficient counts to give a standard error of ±2% were taken.

Isotope dilutions

Hippuric acid. This acid (0·5–2 g.) was added to 2–70 g. of homogenized excreta according to the amount of radioactivity expected. The mixture was warmed to dissolve the

* Part 81: Parke & Williams (1960).

acid and then kept at 0° overnight. The mixture was then filtered and the solid residue dissolved in hot ethanol (in the presence of charcoal) and filtered. The hippuric acid that crystallized on cooling was repeatedly recrystallized from water, ethanol and then from mixtures of ethanol and light petroleum (b.p. 60–80°) to constant activity. With hen excreta, repeated recrystallization failed to remove the radioactivity completely, although radioautographs did not reveal any hippuric acid in hen excreta. Paper chromatography in ethylmethylketone–2N-NH_3 soln. (1:1) indicated that a small amount of ornithuric acid remained in the hippuric acid despite repeated crystallizations. The hippuric acid was finally purified by a large-scale paper chromatogram (Whatman no. 3 paper) with the above-mentioned solvent. The hippuric acid was eluted from the paper with ethanol; the eluate was evaporated to dryness on the water bath and the residue was recrystallized from water and ethanol–ethyl acetate mixtures. It was finally converted into methyl hippurate with ethereal diazomethane and then into hippuramide, m.p. 178°. By this procedure it was possible to show that no radioactive hippuric acid was excreted by hens dosed with [^{14}C]benzoic acid.

Benzoyl glucuronide. Radioautographs of paper chromatograms of hen and pigeon excreta showed that, after feeding with [^{14}C]benzoic acid, little or no free benzoic acid was excreted. Benzoyl glucuronide in the excreta was therefore estimated as benzoic acid after mild alkaline hydrolysis. The homogenized excreta (1–10 g.) were heated on a boiling-water bath for 1 hr. with 1–10 ml. of N-NaOH. Then carrier benzoic acid (0·5–1 g.) was added and the mixture cooled. The mixture was acidified with 2N-H_2SO_4 and cooled to 0°. The crystals which separated were dissolved in water, treated with charcoal and filtered. The benzoic acid, m.p. 122°, which separated was crystallized to constant activity from water, ethanol and light petroleum (b.p. 60–80°). It was further purified as the benzylamine salt, m.p. 126°, which was recrystallized from ethyl acetate.

Total benzoic acid. The homogenized excreta (10 g.) were heated for 3 hr. under reflux with 10N-NaOH (10 ml.). After cooling, carrier benzoic acid (1 g.) was added and the mixture was acidified with 30% (v/v) H_2SO_4. After keeping at 0°, the benzoic acid which separated was purified and counted as above.

Ornithuric acid. Carrier L(+)-ornithuric acid (0·5–1 g.) was dissolved in the minimum amount of N-NaOH and the solution was added to the homogenized excreta (1–3 g.). Conc. HCl was added to the mixture until the pH was about 3. The mixture was cooled and the material which separated was collected and washed with boiling water (50 ml.). This material was recrystallized (after treatment with charcoal) from ethanol–ethyl acetate (1:1) to constant activity. The ornithuric acid had m.p. 186–188°. It was converted into methyl ornithurate by treating it in methanol with ethereal diazomethane. The methyl ester, m.p. 142°, was recrystallized from ethanol or methanol to constant activity.

Paper chromatography and colour reactions. The R_F values of the relevant compounds are given in Table 1. Benzoic acid, benzoyl glucuronide, hippuric acid and ornithuric acid have a light-absorption band or an inflexion at 270–275 mμ (Baldwin *et al.* 1959). When spots containing these compounds on filter paper are examined under a Hanovia Chromatolite lamp, which emits u.v. light of wavelength about 270 mμ, they appear as dark areas.

N^2- and N^5-Benzoyl-L-ornithine are readily detected (10 μg.) on paper by spraying with a 1% solution of ninhydrin in ethanol and then heating at 100–120° for 1 min., when they show up as purple spots. No other compound in Table 1 gives this test. However, the R_F values of these compounds are very similar, but they could be separated in solvent D by allowing the chromatogram to run for 10 hr.

Table 1. *R_F values and colour reactions of benzoic acid and its derivatives*

Whatman no. 4 paper and the descending technique were used. The R_F values were obtained after the front had moved approx. 30 cm. from the origin. The solvents were: A, butanol–acetic acid–water (4:1:5); B, butanol–benzene–water (5:2:2); C, butanol–NH_3 soln. (sp.gr. 0·88)–water (4:1:5); D, ethylmethylketone–2N-NH_3 soln. (1:1); E, propanol–NH_3 soln. (sp.gr. 0·88) (7:3); F, benzene–acetic acid–water (1:1:2); all ratios are by volume. With A, B, C and E the chromatograms were run for 5–6 hr., and with D and F for 2–2·5 hr. All spots showed quenching in ultraviolet light of 270 mμ wavelength.

	R_F						Colour reactions		
Solvent system ...	A	B	C	D	E	F	Ninhydrin	Dimethylaminobenzaldehyde	Dimethylaminocinnamaldehyde
Benzoic acid	1·0	0·95	0·5	0·1	0·65	0·9	.	.	.
Benzoyl glucuronide*†	0·7	0·15	0·25	0·05–0·1	0·35	0	.	.	.
Ornithuric acid	0·9–1·0	0·7	0·65	0·4	0·8–0·95	0·3	.	.	White spot on yellow background
Hippuric acid	0·85	0·2	0·3–0·4	0·1–0·2	0·05	0·1	.	Orange	Red
N^5-Benzoylornithine	0·5	0·1	0·35	0·01†	0·6	0	Purple	.	White spot on yellow background
N^2-Benzoylornithine	0·46	0·1	0·27	0·01†	0·6	0	Purple	.	White spot on yellow background

* Detected by naphtharesorcinol spray (see Baldwin, Robinson & Williams, 1959). † See text.

instead of 2 hr., as given in Table 1. During this longer time the solvent front had run off the paper and the N^5 compound had run 7 cm. from the origin and the N^2 compound, 10 cm. Whenever monobenzoylornithines were suspected to be present this technique was used.

Hippuric acid (10 μg.) is readily detected by spraying the paper with a 1% solution of *p*-dimethylaminobenzaldehyde in acetic anhydride and then heating at 120° for 5 min. Hippuric acid shows up as an orange spot, but none of the other compounds gives a colour.

Ornithuric acid and hippuric acid (10 μg.) are readily differentiated with a solution of *p*-dimethylaminocinnamaldehyde (0·05%, w/v), in acetic anhydride. After being sprayed with this solution the paper was heated in an oven at 12–140° for 2 min. Hippuric acid shows up as a red spot. Ornithuric acid shows up as a white spot on a yellow background and if the spot is viewed under u.v. light it shows an intense bright-blue fluorescence, and the spot is stable for several weeks. Ornithuric acid is detectable by this method in amounts of 10 μg.

RESULTS

Hens. Isotope-dilution experiments for two hens are shown in Table 2. Chromatography and radioautography of hen urine showed that only two metabolites were excreted after the administration of benzoic acid, and these were benzoyl glucuronide and ornithuric acid. The examination of the excreta of six hens which had received benzoic acid and one which had received *p*-aminobenzoic acid failed to produce any evidence that hippuric acid or *p*-aminohippuric acid is formed by hens. The isolation of benzoyl glucuronide from hen excreta has been described by Baldwin *et al.* (1959).

Benzamide is also converted into benzoyl glucuronide and ornithuric acid in hens. A hen (1·4 kg.) was given benzamide (0·34 g.) by mouth. The 24 hr. urine (72 ml.) gave a positive naphtharesorcinol reaction and reduced Benedict's reagent. Determination of the glucuronic acid output showed that 42% of the benzamide had been converted into a glucuronide. Chromatography of the urine showed that neither unchanged benzamide nor free benzoic acid was present. The only compounds detected were benzoyl glucuronide and ornithuric acid. Extraction of half of the urine with ether (see section on turkeys) yielded benzoyl glucuronide (8 mg.; m.p. and mixed m.p. 187°, triacetyl methyl ester, m.p. 138°) and ornithuric acid (10 mg.; m.p. and mixed m.p. 187°).

Pigeons. Isotope-dilution experiments with pigeons are recorded in Table 2. The supernatant fluid obtained by centrifuging the excreta as described above was chromatographed on Whatman no. 4 paper with five solvents (A, B, C, D and E in Table 1). Radioautographs were prepared and the papers were also examined by colour reactions. No spot corresponding to ornithuric acid was found. Free benzoic acid and the monobenzoylornithines were not found in the urine.

An adult pigeon was given 135 mg. of benzoic acid by mouth and its excreta were collected for 24 hr. The excreta were mixed with dilute ammonia solution (5 ml.) and ethanol (5 ml.) and then centrifuged. The supernatant, after a 2 ml. portion had been removed for chromatography, was acidified with 2N-H_2SO_4 and continuously extracted with ether for 6 hr. The extract was dried with anhydrous Na_2SO_4 and evaporated. The residue was extracted with ether (10 ml.) and the extract was again evaporated to dryness. The second residue was now treated with light petroleum (b.p. 60–80°; 5 ml.) to remove fatty materials and the residue was recrystallized from water. Hippuric acid (2 mg.) was obtained with m.p. and mixed m.p. 187°.

Muscovy ducks. Two ducks, a male (2·5 kg.) and a female (2 kg.), were each given 2 g. of benzoic acid by crop tube, and the urine was collected for 24 hr. The urine reduced Benedict's reagent and gave a strong naptharesorcinol reaction. Paper chromatography of the urine in solvents A, B, C and D (see Table 1) showed the presence of benzoyl glucuronide and ornithuric acid and the absence of hippuric acid.

Turkeys. Two female turkeys (6 kg. each) were each given 6 g. of benzoic acid on 2 successive days and the excreta were collected for 3 days. As the birds ate little food during the experiment, the excreta contained only small amounts of solid material. Paper chromatography showed that the urine contained no hippuric acid, but large amounts of benzoyl glucuronide and ornithuric acid. The urine was centrifuged to remove solid debris and then brought to pH 2–3 with conc. HCl. It was then continuously extracted with ether for 4 days, a little ethanol being added from time to time to break up emulsions of urine and ether and the ether being changed daily. The ether extracts were evaporated *in vacuo* and the residue was dried overnight in a vacuum desiccator containing

Table 2. *Fate of* [^{14}C]*benzoic acid in hens and pigeons*

Benzoic acid was administered orally.

	Hens (Leghorns)		Pigeons	
Expt. no.	H1	H2	P1	P2
Wt. of bird (kg.)	1·85	1·65	0·3	0·3
Dose of benzoic acid (mg./kg.)	243	242	209	181
Dose of ^{14}C (μc/bird)	8·5	14·4	15·7	4·0
Metabolites excreted (% of dose)				
Time after dosing (hr.)	24	24	18·5	18·5
Benzoyl glucuronide	26·3	32·2	28·8	25·1
Ornithuric acid	47·2	40·3	0·0	0·0
Hippuric acid	0·0	0·0	63·2	60·8
Sum of conjugates	73·5*	72·5	92·0	85·9
Total benzoic acid	86·3*	74·5	94·8	82·6
Total ^{14}C in urine	89·0	71·6	93·0	81·0
^{14}C in urine after 48 hr.	89·0	78·0	94·0	88·0

* The agreement here is not as good as in the other columns, but no compounds were found other than benzoyl glucuronide and ornithuric acid.

P_2O_5 and anhydrous Na_2CO_3. The residue was taken up in the minimum quantity of ethanol and 10 vol. of $CHCl_3$ added. On keeping at 0°, crude benzoyl glucuronide (5·4 g.) separated. Two recrystallizations from ethanol (after treatment with charcoal) gave 1·9 g. of pure benzoyl glucuronide, m.p. 187° and $[\alpha]_D^{27}$ −25·6° (*c*, 2·5 in water). The glucuronide was further characterized as the methyl ester, m.p. 191·5° and $[\alpha]_D^{27}$ −16·1° (*c*, 1 in methanol) and the triacetyl methyl ester, m.p. 145° and $[\alpha]_D^{26}$ −15° (*c*, 1 in $CHCl_3$).

The $CHCl_3$ filtrate from the benzoyl glucuronide was evaporated to dryness and the residue extracted with ether (200 ml.). This extract was discarded and the residue was shaken with water (50 ml.) to remove any remaining glucuronide. The residue remaining was now taken up in ethanol (100 ml.) and the solution treated with ether (300 ml.). On keeping for several days L-ornithuric acid (m.p. 182°; 1·2 g.) separated. It was purified by dissolving in ethanol and then adding ether and had m.p. 187° and $[\alpha]_D^{22·5}$ +9·8° (*c*, 1 in N-NaOH). A further crop of 0·5 g., m.p. 184° was obtained by treatment of the mother liquors from the first crop.

Wood pigeon. The wood pigeon (450 g.) was given a pellet of benzoic acid (150 mg.) and its excreta were collected for 24 hr. Benzoyl glucuronide and hippuric acid were shown by paper chromatography to be present in the excreta. No ornithuric acid was found. The excreta were homogenized and brought to pH 3 with conc. HCl and then continuously extracted with ether for 8 hr. Evaporation of the ether left white crystals, which on recrytallization from water (after treatment with charcoal) gave 53 mg. of hippuric acid, m.p. and mixed m.p. 188°.

Barbary dove. The dove (180 g.) was given a pellet of benzoic acid (47 mg.). Paper chromatography of the 24 hr. excreta showed the presence of hippuric acid but not of ornithuric acid. The excreta gave a weak naphtharesorcinol test for glucuronic acid.

Carrion crow. The crow (500 g.) was given a capsule containing 240 mg. of benzoic acid on one day, and a second capsule containing 220 mg. on the succeeding day. The excreta were collected during 3 days. Chromatography of the excreta showed the presence of benzoyl glucuronide, but neither hippuric acid nor ornithuric acid was found. By extraction of the homogenized excreta at pH 3 with ether as described above, benzoyl glucuronide (100 mg.) was isolated, which, after recrystallization, had m.p. 179°, not depressed by the authentic compound, m.p. 184°. It was further identified by its positive naphtharesorcinol reaction and reduction of Benedict's reagent on warming.

Goose. A domestic goose (3·5 kg.) was given 1·5 g. of benzoic acid as small pellets. The 24 hr. urine contained benzoyl glucuronide and ornithuric acid, but no hippuric acid, as shown by paper chromatography. Extraction of the acidified urine yielded L(+)-ornithuric acid (40 mg. after purification), m.p. 184°, not depressed by an authentic sample.

Parrot. A grey African parrot (350 g.) was given 170 mg. of sodium benzoate in water by subcutaneous injection. It could not be persuaded to take benzoic acid by mouth. The clear urine was collected for 6 hr. The urine gave a strong naphtharesorcinol reaction and readily reduced Benedict's reagent on warming. Paper chromatography of the urine showed the presence of benzoyl glucuronide, but neither ornithuric acid nor hippuric acid was found.

Destruction of ornithuric acid by chicken faeces

The quantitative results for hens quoted in Table 2 were obtained when the urine and a small amount of faeces passed during 24 hr. after dosing were collected in a vessel containing 2 ml. of saturated aqueous mercuric chloride. The use of mercuric chloride became necessary when it was discovered that chicken faeces could destroy ornithuric acid. In one experiment in which no mercuric chloride was used, and in which there was gross faecal contamination of the urine, the ornithuric acid output estimated by isotope dilution amounted to only 0·5 % of the dose (2 m-moles/kg. of [^{14}C]benzoic acid). During this particular experiment samples of urine had been collected by catheter at 4 and 6 hr. after dosing. On chromatography of these urines on paper, only two spots were found by radioautography and colour reactions and these corresponded to ornithuric acid and benzoyl glucuronide. The rest of the urine was allowed to mix with the faeces passed, and the mixture was examined 4 days later. Paper chromatography of this mixture gave spots corresponding to benzoyl glucuronide, benzoic acid and N^5-benzoylornithine and a weak ornithuric acid spot. The N^5-benzoylornithine was readily detected by its radioactivity and by ninhydrin. This experiment suggested that the fresh chicken urine contained ornithuric acid, but no monobenzoylornithines, and that chicken faeces were capable of breaking down ornithuric acid. Chicken faeces and urine were therefore examined qualitatively for 'ornithuricase' activity.

Fresh chicken urine was collected by catheterization and 1 ml. was diluted to 10 ml. with 0·067 M-phosphate buffer, pH 7. Fresh chicken faeces (1 g. wet wt.) was made into a suspension of 10 ml. with the same buffer. A solution of L(+)-ornithuric acid containing 2 mg./ml. was made up in the same buffer. The diluted faeces or urine (1 ml.) was mixed with the ornithuric acid solution (1 ml.) and incubated at 35°. The controls consisted of 1 ml. of water plus 1 ml. of ornithuric acid solution, and 1 ml. of boiled faeces or urine (boiled for 30 min.) plus 1 ml. of the ornithuric acid solution. At suitable intervals 0·1 ml. samples of the

solutions were chromatographed on Whatman no. 4 paper with solvent D (see Table 1).

It was found that chicken urine had no apparent action on ornithuric acid at pH 7 even after a week's incubation. The faeces (0·1 g.) destroyed the ornithuric acid (2 mg.) within 21 hr., and although benzoic acid was detectable in the mixture, neither ornithuric acid nor monobenzoylornithines were present. If the faeces were boiled for 30 min., then they had no action on ornithuric acid. However, if the faeces were boiled for only 0·5 min., they still destroyed ornithuric acid, but N^5-benzoylornithine was readily detected in the incubated mixture after 21 and 28 hr., but after 48 hr. it also had disappeared.

The suspension of faeces (10 ml.) was centrifuged at 4500 rev./min. for 15 min. and the supernatant was used as an enzyme sample. The solid residue was washed with water (2 × 10 ml.) and again centrifuged, and the residue was finally suspended in 10 ml. of phosphate buffer. The untreated supernatant and samples after boiling for ½ min. and 30 min. were incubated with ornithuric acid solutions as described above. The unboiled supernatant destroyed the ornithuric acid in 21 hr. at which time N^5-benzoylornithine was readily detected. The supernatant boiled for ½ min. was less active and ornithuric acid was still present after 21 hr., but after 68 hr. N^5-benzoylornithine became detectable. The supernatant boiled for 30 min. was inactive. The suspension (1 ml.) of the solid residue from the centrifuged faeces was also incubated as described above with the ornithuric acid solution (1 ml.). It readily destroyed ornithuric acid within 21 hr. and although benzoic acid could be detected in the incubated mixture, no monobenzoylornithines were found. If the suspension were boiled for 0·5 min., it still destroyed the ornithuric acid within 21 hr., but now both benzoic acid and N^5-benzoylornithine were found in the incubated mixture. Boiling the suspension for 30 min. destroyed its 'ornithuricase' activity. This activity is completely inhibited by aqueous 0·035 % mercuric chloride.

These experiments show that chicken faeces, but not the urine, can destroy ornithuric acid and that the activity is mainly associated with the solid particles of the faeces. They also suggest that stepwise removal of benzoyl groups from ornithuric acid may occur, the N^2-benzoyl group being removed first, as N^2-benzoylornithine was never detected in any of the experiments.

Further experiments suggested that the faeces destroyed L(+)-ornithuric acid but not the D-isomer, for when DL-ornithuric acid was incubated with fresh faeces at pH 7, benzoic acid and N^5-benzoylornithine were found within 24 hr., but an ornithuric acid spot was found on the chromatograms even after incubation for 3 days and this is due presumably to D-ornithuric acid. In parallel incubations with L(+)-ornithuric acid, the acid had always completely disappeared within 24 hr. The faeces had little action on di-(*o*-hydroxybenzoyl)- and di-(*p*-aminobenzoyl)-L(+)-ornithines or hippuric acid.

DISCUSSION

It has been assumed in the past that in birds aromatic acids are excreted conjugated with ornithine, and this assumption has been based largely on observations on the domestic fowl. Tables 2 and 3 show that this assumption is incorrect because, of the nine species of bird examined, only four excreted ornithuric acid in response to ingested benzoic acid. What is common to them all is the excretion of benzoyl glucuronide. Of the orders examined, only the galliformes and anseriformes were found to form ornithuric acid. The columbiformes, which include the pigeons and doves, excreted hippuric acid and not ornithuric acid. The other species examined, which included a carrion crow and a parrot, excreted neither ornithuric acid nor hippuric acid and require further investigation.

Table 2 shows the quantitative aspects of the fate of benzoic acid in the domestic hen and pigeon. In both species benzoyl glucuronide accounts for about a quarter of the dose. In the hen, however, ornithuric acid accounts for 40–50 % of the dose and there is no hippuric acid excreted. In the pigeon, on the other hand, no ornithuric acid is formed, and about 60 % of the dose is excreted as hippuric acid. In both species about 70–90 % of

Table 3. *Metabolites of benzoic acid in some species of birds*

+ Indicates present and −, absent.

	Metabolites isolated or detected		
	Benzoyl glucuronide	Hippuric acid	Ornithuric acid
Galliformes			
Domestic fowl	+	−	+
Turkey	+	−	+
Anseriformes			
Muscovy duck	+	−	+
Goose	+	−	+
Columbiformes			
Pigeon	+	+	−
Wood pigeon	+	+	−
Barbary dove	+	+	−
Passeriformes			
Carrion crow	+	−	−
Psittaciformes			
Grey African parrot	+	−	−

the dose of benzoic acid is eliminated in 24 hr. after dosing. It is known that glycine is necessary for optimum growth in chickens and turkeys, but the glycine requirement of other birds is not known. It has been suggested that this requirement of glycine may be related to the biosynthesis of uric acid, which is a major nitrogenous waste product in birds, including the pigeons (see Arnstein, 1954). It is possible that the formation of hippuric acid is peculiar to the columbiformes, but many more species of birds have to be examined before it could be claimed that hippuric acid formation is characteristic of columbiformes.

SUMMARY

1. Benzoic acid has been administered to nine species of birds, namely the domestic fowl, turkey, goose, Muscovy duck, domestic pigeon, wood pigeon, Barbary dove, carrion crow and grey African parrot.

2. All these species of birds excrete benzoyl glucuronide.

3. The domestic fowl, turkey, goose and Muscovy duck also excreted L(+)-ornithuric acid but no hippuric acid.

4. The domestic pigeon, wood pigeon and Barbary dove also excreted hippuric acid but no ornithuric acid.

5. The carrion crow and grey African parrot did not appear to excrete hippuric acid or ornithuric acid.

6. [*carboxy*-^{14}C]Benzoic acid was administered to hens and pigeons. The hens excreted about 25–30 % of the dose (240 mg./kg.) as glucuronide and 40–50 % as ornithuric acid in 24 hr. The pigeons excreted 25–30 % of the dose (200 mg./kg.) as glucuronide and 60 % as hippuric acid in 18·5 hr.

7. Chicken faeces contain an enzyme which decomposes L(+)-ornithuric acid via N^5-benzoylornithine. Monobenzoylornithines were not found as metabolites of benzoic acid in hens.

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REFERENCES

Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 16.
Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.
Barrass, B. C. & Elmore, D. T. (1957). *J. chem. Soc.* p. 3134.
Jaffe, M. (1877). *Ber. dtsch. chem. Ges.* **10**, 1925.
Karrer, P. & Ehrenstein, M. (1926). *Helv. chim. acta*, **9**, 323.
McGilvery, R. W. & Cohen, P. P. (1950). *J. biol. Chem.* **183**, 179.
Parke, D. V. & Williams, R. T. (1960). *Biochem. J.* **74**, 5
Robinson, D. & Williams, R. T. (1958). *Biochem. J.* **68**, 23P.
Schachter, D., Manis, J. G. & Taggart, J. V. (1955). *Amer. J. Physiol.* **182**, 537.
Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed. p. 348. London: Chapman and Hall Ltd.

Biochem. J. (1960) **76**, 600

Studies in Detoxication

83. THE AROMATIZATION OF *CYCLO*HEXANECARBOXYLIC ACID IN HENS

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*cyclo*Hexanecarboxylic acid (hexahydrobenzoic acid) is aromatized to benzoic acid to a considerable extent in man, the rabbit and the dog (Bernhard & Caflisch-Weill, 1945) and to a minor extent in the rat (Beer, Dickens & Pearson, 1951). In man and the rabbit the main excretory product of *cyclo*hexanecarboxylic acid is hippuric acid. This aromatization reaction has also been studied in tissue slices and homogenates (Beer *et al.* 1951; Mitoma, Posner & Leonard, 1958) and the last-named authors found that aromatization took place in guinea-pig- and in rabbit-liver mitochondria in the presence of adenosine triphosphate and glycine. A very weak aromatizing activity was found in rat-liver mitochondria, but no activity was found with liver mitochondria from man, cat, mouse, dog, monkey, toad, pigeon and chicken. This lack of activity was attributed to the lability of the enzyme. With chicken mitochondria replacement of glycine by ornithine did not produce aromatization. Furthermore, there is no evidence in the literature that the intact chicken can aromatize *cyclo*hexanecarboxylic acid. In the present paper it will be shown that chickens can

aromatize *cyclo*hexanecarboxylic acid and that the main excretory products are benzoyl glucuronide and ornithuric acid.

MATERIALS AND METHODS

Materials. *cyclo*Hexanecarboxylic acid (L. Light and Co. Ltd). had b.p. 230° after distillation. The amide, m.p. 188°, was prepared via the acid chloride (cf. Lumsden, 1905).

cyclo[*carboxy*-^{14}C]Hexanecarboxylic acid was prepared from [*carboxy*-^{14}C]benzoic acid according to the method of Mitsubara & Perkin (1905). The benzoic acid (4 g., 7 μc/g.) in boiling *iso*amyl alcohol (200 ml.) was reduced with sodium (20 g.) and the procedure of Mitsubara & Perkin (1905) followed exactly to the stage where the *cyclo*hexanecarboxylic acid is purified by distillation. Instead of distillation, the acid in 20 ml. of ether was treated with 3·6 g. of benzylamine. The benzylamine salt which separated was then recrystallized to constant activity (yield 1·2 g. containing 3·56 μc/g.). For feeding experiments the whole of this salt was dissolved in water (60 ml.) with warming, and the solution treated with conc. HCl (5 drops) and extracted with ether (3 × 100 ml.). The extract was dried over anhydrous $CaCl_2$ and then evaporated in a current of warm air. This gave 0·47 g. of *cyclo*hexanecarboxylic acid (b.p. 229°), which solidified on cooling.

Other compounds used are described in the preceding paper (Baldwin, Robinson & Williams, 1960).

Hens. The hens used were Brown Leghorns. The *cyclo*hexanecarboxylic acid was administered by crop tube in water containing just sufficient $NaHCO_3$ to dissolve the acid. The hens were deprived of food the evening before administration of the compound, and the urine was collected in flasks containing 2 ml. of saturated aqueous mercuric chloride (see Baldwin *et al.* 1960).

Isotope methods. Benzoyl glucuronide and ornithuric acid were determined as described by Baldwin *et al.* (1960).

cyclo*Hexanecarboxylic acid.* The urine (20–40 ml.) was heated under reflux for 3 hr. with an equal volume of 40% (w/v) NaOH. To the cooled solution *cyclo*hexanecarboxylic acid (0·6–0·9 g.) in a little N-NaOH was added, and the solution was then neutralized with 30% (w/v) H_2SO_4 and extracted with ether. The extract was dried and the *cyclo*hexanecarboxylic acid was precipitated as its benzylamine salt, which was recrystallized from ethyl acetate to constant activity. The salt was reconverted into the free acid, which was methylated with ethereal diazomethane. The ethereal solution of the methyl ester was then saturated with dry ammonia, and the *cyclo*hexanecarboxylic acid amide was recrystallized from water (m.p. 188°) to constant activity.

Chromatography. The methods used are described by Baldwin *et al.* (1960).

RESULTS

Detection of benzoyl glucuronide and ornithuric acid

A portion of the urine from the hens which had received the *cyclo*[^{14}C]hexanecarboxylic acid was acidified with strong HCl and extracted with ethyl acetate. The extract was concentrated to a small volume and suitable amounts were chromatographed on Whatman no. 4 paper in solvent systems A, C and D of Baldwin *et al.* (1960). These chromatograms were left in contact with X-ray film for 14 days. The resulting radioautographs showed spots corresponding in R_F values to benzoyl glucuronide and ornithuric acid in the three solvent systems. Hippuric acid was not found. Chromatograms were also prepared, in solvent systems A, B, C, D and F of Baldwin *et al.* (1960), from the urine of hens dosed with non-radioactive *cyclo*hexanecarboxylic acid. Only benzoyl glucuronide and ornithuric acid were detected in these urines by R_F values and colour reactions.

Isolation of metabolites

Benzoic acid. A hen (wt. 1·65 kg.) was given orally 1 g. of *cyclo*hexanecarboxylic acid as the sodium salt in water. The liquid excreta (130 ml.) were collected for 24 hr. and were boiled (reflux) for 3 hr. with 40% NaOH (65 ml.). After cooling, the mixture was acidified with 50% (v/v) H_2SO_4 and steam-distilled until 1·5 l. of distillate had been collected. The distillate was extracted with 2 × 250 ml. of ether, and the extract dried over anhydrous $CaCl_2$ and then evaporated in a current of cold air to yield 800 mg. of benzoic acid, m.p. 116°. This corresponded to 88% of the dose of *cyclo*hexanecarboxylic acid. On recrystallization from water and then from light petroleum, pure benzoic acid, m.p. and mixed m.p. 121°, was obtained. This was confirmed by preparing the benzylamine salt, m.p. and mixed m.p. 127°.

Benzoyl glucuronide. A total of 6 g. of *cyclo*hexanecarboxylic acid was fed to six hens. The 24 hr. urine was acidified to pH 2–3 with conc. HCl and then continuously extracted with ether for 16 hr. After drying over anhydrous Na_2SO_4 the extract was evaporated. The residue was taken up in ethanol (10 ml.) and light petroleum (b.p 60–80°) added. Benzoyl glucuronide (120 mg.) separated and after three recrystallizations from ethanol–light petroleum mixtures had m.p. and mixed m.p. 186° and $[\alpha]_D^{23}$ – 23·6° (c, 1 in water) [light absorption, $\lambda_{max.}$ 230 and 274 mμ with corresponding $\epsilon_{max.}$ 12 400 and 1120 in ethanol; see Baldwin, Robinson & Williams (1959)].

L(+)-*Ornithuric acid.* A hen that had been starved for 17 hr. was given orally 2 g. of *cyclo*hexanecarboxylic acid

Table 1. *Metabolites of* cyclo[^{14}C]*hexanecarboxylic acid in the hen*

For details see text.

Expt. no.	1	2
Wt. of hen (kg.)	1·5	1·8
Dose of acid (mg./kg.)	260	205
Dose of ^{14}C (μc/hen)	0·77	1·57
Metabolites excreted in 24 hr. (% of dose)*		
Benzoyl glucuronide	49	57
Ornithuric acid	17	11
*cyclo*Hexanecarboxylic acid	6	3
Sum of metabolites	72	71
^{14}C in 24 hr. urine	82	67
^{14}C in 48 hr. urine	82	69

* Determined by isotope dilution.

as the sodium salt in water. The 24 hr. urine was acidified and extracted with ether as above. The extract was dried and evaporated as before and the residue was treated with 100 ml. of $CHCl_3$ at 60° and the extract filtered. Evaporation of the $CHCl_3$ gave a clear gum, which was taken up in 10 ml. of ethanol and then 30 ml. of water was added. On keeping overnight L(+)-ornithuric acid (80 mg.) separated. It had m.p. 184°, not lowered by the authentic acid, and $[\alpha]_D^{23}$ +5·5° (*c*, 1·3 in 0·1 N-NaOH) (light absorption in 0·1 N-HCl, $\lambda_{max.}$ 229 mμ, $\epsilon_{max.}$ 20 100 with a slight inflexion at 270 mμ).

The results of the isotope-dilution experiments are shown in Table 1.

DISCUSSION

The aromatization of *cyclo*hexanecarboxylic acid has been shown to occur in guinea-pig-liver mitochondria and the end product of the process is hippuric acid. Apparently the process takes place in three steps, the first being the activation of *cyclo*hexanecarboxylic acid by formation of *cyclo*hexanecarbonyl-coenzyme A, the second the dehydrogenation of *cyclo*hexanecarbonyl-coenzyme A to benzoyl-coenzyme A, and the third the formation of hippuric acid by the reaction of benzoyl-coenzyme A with glycine. In the absence of glycine, aromatization occurs only to a very minor extent (Mitoma *et al.* 1958), for removal of the aromatization product, benzoic acid, as hippuric acid greatly accelerates the aromatization step. This process was also studied in the liver mitochondria of two birds, the pigeon and the hen, but Mitoma *et al.* (1958) were unable to show aromatization in these species, even when glycine was replaced by ornithine in the system used. The present work shows that the intact hen can aromatize *cyclo*hexanecarboxylic acid very efficiently and that the main product is benzoyl glucuronide. Table 1 shows that 70–80 % of the metabolites (50–60 % of the dose) of *cyclo*hexanecarboxylic acid consist of benzoyl glucuronide and about 20 % (10–20 % of the dose) of ornithuric acid.

Mitoma *et al.* (1958) were also unable to detect aromatization of *cyclo*hexanecarboxylic acid in the liver mitochondria of the cat, mouse, dog, monkey and human, although aromatization is known to occur in man and the dog *in vivo*. It is possible that aromatization might have been detected in the mitochondria of some of these species if glycine had been replaced by a source of biologically active glucuronic acid such as uridine diphosphate glucuronic acid, which would have allowed the benzoic acid to be removed as glucuronide.

SUMMARY

1. *cyclo*[*carboxy*-^{14}C]Hexanecarboxylic acid has been prepared by the reduction of [*carboxy*-^{14}C]-benzoic acid.

2. *cyclo*Hexanecarboxylic acid is extensively aromatized when administered orally to hens. This was proved by the isolation of benzoyl glucuronide and L(+)-ornithuric acid from the urine. By isotope dilution it is shown that of the ^{14}C administered orally to hens as *cyclo*hexanecarboxylic acid (dose 200–260 mg./kg.) about 80 % is excreted in 24 hr.; 50–60 % of the dose is excreted as benzoyl glucuronide and 10–20 % as ornithuric acid. About 5 % of the *cyclo*hexanecarboxylic acid is excreted unchanged.

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REFERENCES

Baldwin, B. C., Robinson, D. & Williams, R. T. (1959). *Biochem. J.* **71**, 638.

Baldwin, B. C., Robinson, D. & Williams, R. T. (1960). *Biochem. J.* **76**, 595.

Beer, C. T., Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 222.

Bernhard, K. & Caflisch-Weill, H. (1945). *Helv. chim. acta*, **28**, 1697.

Lumsden, J. S. (1905). *J. chem. Soc.* **87**, 90.

Mitoma, C., Posner, H. S. & Leonard, F. (1958). *Biochim. biophys. Acta*, **27**, 156.

Mitsubara, K. & Perkin, W. H. (1905). *J. chem. Soc.* p. 661.

The Formation *in vivo* of Lombricine in the Earthworm (*Megascolides cameroni*)

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Lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) was first isolated from earthworms (*Lumbricus terrestris*) by Van Thoai & Robin (1954), who held the view that *N*-phosphoryl-lombricine was the phosphagen of that species. Subsequently it was suggested that the biological precursor of lombricine was serine ethanolamine phosphodiester (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, II) and that lombricine was probably formed by the transfer of the amidino group of arginine (III) to serine ethanolamine phosphodiester (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959). This suggestion has received support from: (*a*) the discovery of serine ethanolamine phosphodiester in earthworms (Rosenberg & Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960); (*b*) the finding that the serine moiety of serine ethanolamine phosphodiester (Ennor *et al.* 1960), like that of lombricine (Beatty, Magrath & Ennor, 1959), has the unusual D-configuration, and (*c*) the demonstration that after the administration of inorganic ^{32}P to earthworms, the specific radioactivity of serine ethanolamine phosphodiester was much greater than that of lombricine (Gaffney, Rossiter, Rosenberg & Ennor, 1960).

This paper describes experiments in which [1:2-$^{14}C_2$]ethanolamine, DL-[3-^{14}C]serine, L-[^{3}H]-serine and D-[^{3}H]serine were administered to earthworms. It also provides additional evidence that serine ethanolamine phosphodiester is the precursor of lombricine and that the amidino group of the latter is derived from the amidino group of arginine, probably by a transfer of this group to serine ethanolamine phosphodiester with the production of ornithine (IV), according to the transamidinase reaction in Scheme 1.

A preliminary account of these experiments has already appeared (Rossiter, Gaffney, Rosenberg & Ennor, 1960).

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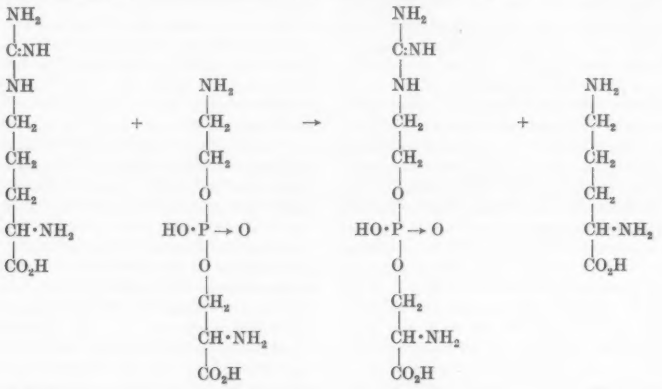
MATERIALS AND METHODS

Reagents. All reagents were analytical grade, and the ethanol and butanol used in chromatographic solvents were redistilled before use. Serine ethanolamine phosphodiester



Scheme 1

(SEP) was synthesized by Beatty & Magrath (1959) after the method of Jones & Lipkin (1956). Lombricine was prepared from earthworms as described by Ennor *et al.* (1960).

Radioactive precursors. [1:2-$^{14}C_2$]Ethanolamine and DL-[3-^{14}C]serine were obtained from The Radiochemical Centre, Amersham, Bucks, and were used at specific radioactivities of 5 and 2·7 μc/μmole respectively.

L-Serine and D-serine, obtained from commercial sources, were tritiated according to the process of Wilzbach (1957) by The Radiochemical Centre. In order to remove possible decomposition products and exchangeable tritium the tritiated material (100 mg. in each instance) was dissolved in 20 ml. of water and applied to a column (1 cm. × 10 cm.) of Amberlite CG-120 (mesh 100–200, H^+ form) ion-exchange resin. The column was eluted with a large volume of water, which was collected in 50 ml. fractions. Those fractions containing serine were reapplied to a Zeo-Karb 225 (H^+ form) column (2 cm. × 10 cm.) and the adsorbed material was eluted with aq. 2·5N-NH_3 solution. The eluate was dried under reduced pressure at a temperature of 40°. Sufficient water and non-radioactive L- or D-serine were added to make a solution with a final concentration of 200 μmoles/ml. and a specific radioactivity of 13·5 μc/μmole.

L-[*amidino*-^{14}C]Arginine was prepared from $Na^{14}CN$ (1 mc) and L-ornithine monohydrochloride as described by Stetten & Bloom (1956). The final product had a specific radioactivity of 0·042 μc/μmole.

Animals. Earthworms (*Megascolides cameroni*) of average weight 8 g. were collected in the Australian Capital Territory. They were kept at 10° in cardboard cartons filled with moist earth, and under these conditions were maintained in good condition for many months.

Experimental procedure. The appropriate radioactive precursor (in the amount and with the specific radioactivity listed in the tables) was given to the earthworm from a microsyringe through a fine polythene tube inserted into the anterior end of the gastrointestinal tract for a distance of at least 3 cm. The worm was maintained as described above for periods varying from 24 hr. to 9 days, after which time it was killed and the whole of the gastrointestinal tract and appendages were dissected free from the muscular body wall. These two tissues were treated separately, and are referred to as 'gut' and 'muscle' respectively.

The tissue samples were rapidly weighed and homogenized with 2 vol. of 1·5N-perchloric acid in the micro-cup of a Nalco homogenizer (MSE, Birmingham). After centrifuging, the supernatant fluid was neutralized with 2·5N-KOH and the precipitated $KClO_4$ was removed by further centrifuging. Ethanol (1 vol.) was added to the supernatant fluid and the mixture was kept at 3° for at least 1 hr. to ensure complete precipitation of glycogen, which was then removed by centrifuging. The supernatant fluid was applied to a column (1 cm. × 6 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form), at a rate of 1 ml./min. Water (approx. 20 ml.) was passed through the column until the pH of the effluent had risen to 5, and the washings and effluent were discarded. The material retained by the resin was then eluted with aq. 2·5N-NH_3 soln. at a rate of 3 ml./min. The eluate (approx. 50 ml.) was dried under reduced pressure at 40° and the residue dissolved in a small volume of water (100 μl. for gut and 300 μl. for muscle). These solutions were used in the chromatographic separation of lombricine, SEP and amino acids, and are referred to in the following section as gut or muscle extracts.

In order to evaluate the extent of re-ingestion of excreted radioactive material given, one worm was injected with a quantity of [^{14}C]serine equal to that given to the experimental animals, and was placed in a box filled with moist earth (400 g.). Another worm, which received no injection, was placed in the same box, and the two worms were left in the earth for 8 days. They were then both killed and each worm was treated as described above. The earth from the box was extracted with perchloric acid and the extract was desalted and reduced in volume. The total ^{14}C present in both worms, and in the soil, was assessed. Of the total radioactivity present, the injected worm was found to contain 86·0%, the earth 13·6% and the control worm 0·5%. Complications through re-ingestion of excreted radioactive material were therefore not considered of importance in the present experiments.

Chromatography. Whatman no. 3 filter paper (56 cm. × 56 cm.) washed with water was used throughout. The papers were handled with plastic forceps to avoid accidental contamination with ninhydrin-reacting substances. For the separation of SEP and lombricine the entire sample of the gut extract or 100 μl. of the muscle extract was applied to the paper between marker spots containing samples of authentic SEP and lombricine. Chromatograms (descending) were run in ethanol–formic acid–water (70:10:20, by vol.) and dried in a current of air at 50°. Lombricine and SEP were located by reference to the marker spots, which were identified by spraying with a 0·2% solution of indanetrione hydrate (ninhydrin) in acetone and heating at 70° for 10 min. In this way it was possible to separate SEP (R_F approx. 0·2) and lombricine (R_F approx. 0·3) from all other ninhydrin-reacting material ($R_F > 0·4$), although in some experiments SEP and lombricine were not completely separated from each other. A complete separation was achieved by applying the eluted samples of SEP or lombricine to a second sheet of filter paper and rechromatographing in phenol–water (4:1, w/v). The SEP (R_F approx. 0·3) and lombricine (R_F approx. 0·55) were identified and eluted as before.

For the separation of free amino acids the entire sample of the gut extract or 100 μl. of the muscle extract was applied to the corner of a sheet of filter paper for two-dimensional chromatography. The first solvent was either ethanol–formic acid–water, as described above, or butanol–water–acetic acid (50:30:20, by vol.). The second solvent was phenol–water (4:1, w/v). In experiments with ^{14}C-labelled precursors the free amino acids, and in some instances SEP and lombricine, were located radioautographically by applying the chromatograms to sheets of Ilford Industrial X-ray film for 2 weeks. Areas on the chromatogram corresponding to darkened areas on the radioautograph were cut out and the material was eluted with water. An alternative procedure for locating the amino acids, which was always used with the ^{3}H-labelled precursors, was the spraying of the chromatogram with a dilute (0·05%, w/v) solution of ninhydrin in acetone, followed by heating to 70° for 10 min. Ninhydrin-positive areas on the chromatogram corresponding to the required amino acids were cut out and the material was eluted. This method effected a considerable saving in time but the colour of the eluates necessitated the use of an internal standard for the scintillation counting.

The amino acids were identified on the chromatograms by the use of reinforcing markers of authentic compounds, or, in cases of doubt, by rechromatographing the material with

appropriate markers in other standard solvents commonly used for the separation of amino acids.

Counting. A measured portion of the eluted sample of lombricine, SEP or amino acid was pipetted into a 25 ml. glass vial and the water evaporated at a temperature not above 50°. The residue was dissolved in 200 μl. of 0·5 M-*p*-*N*-(di*iso*butylcresoxyethoxyethyl)-*NN*-dimethyl-*N*-benzylammonium hydroxide (hydroxide of Hyamine 10-X, Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in dry methanol as described by Passmann, Radin & Cooper (1956) and Vaughan, Steinberg & Logan (1957). To this was added 10 ml. of solvent-scintillator containing 4 g. of 2:5-diphenyloxazole and 100 mg. of 1:4-di-(2:5′-phenyloxazolyl)-benzene/l. of toluene. The radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer model 314 (Packard Instrument Co., La Grange, Ill., U.S.A.) with a 10–50 v window and high-voltage tap 4 for ^{14}C-labelled precursors and tap 8 for ^{3}H-labelled precursors. When the materials were located in the chromatogram with the ninhydrin-detection spray, an internal standard of either [^{14}C]serine or [^{3}H]serine was used to correct for quenching.

Quantitative determination of serine ethanolamine phosphodiester, lombricine and amino acids. The amount of SEP, lombricine or amino acid in the material eluted from the filter paper was determined by the principles of the method of Connell, Dixon & Hanes (1955), in which NH_3, the presence of which greatly reduces the reliability of the procedure, is removed by drying the material in the presence of an alkaline buffer. The ninhydrin colour was then developed under the conditions described by Moore & Stein (1954). Any NH_3 present in the water used for the preparation of all reagents was removed by passing the water through a large column of Zeo-Karb 225 ion-exchange resin (H^+ form) immediately before use.

A measured portion of the eluted material was pipetted into a test tube containing 100 μl. of borate buffer in methanol prepared as described by Connell *et al.* (1955) and the tube left overnight under reduced pressure in a desiccator containing conc. H_2SO_4. To the tube was then added 0·5 ml. of 30% (v/v) acetic acid to give a final pH of 5, which was found optimum for the development of the ninhydrin colour. The amount of ninhydrin-reacting material in the sample was determined as described by Moore & Stein (1954), authentic samples of SEP, lombricine or serine being used as reference standards.

Hydrolysis of lombricine. In many of the experiments the eluted lombricine was separated into its constituent serine and guanidinoethanol moieties by hydrolysis in a sealed Pyrex tube in 6 N-HCl at 110° for 24 hr. After removal of the HCl under reduced pressure in a desiccator containing KOH, the residue was dissolved in 100 μl. of water and applied to a sheet of filter paper between marker spots containing either (*a*) serine and lombricine (detected with ninhydrin), or (*b*) the guanidine derivatives, lombricine, guanidinoethanol and guanidinoethyl phosphate (detected with diacetyl reagent as described by Rosenberg, 1959). The chromatogram was run in phenol–water (4:1, w/v) and dried in the usual way. Under the conditions of the hydrolysis, lombricine was converted into serine (R_F approx. 0·35) and a mixture of guanidinoethanol (R_F approx. 0·8) and guanidinoethyl phosphate (R_F approx. 0·7). Serine and the material on the combined guanidinoethanol and guanidinoethyl phosphate area were eluted and counted as described above.

RESULTS

[1:2-$^{14}C_2$]Ethanolamine. After the administration of [1:2-$^{14}C_2$]ethanolamine the specific radioactivity of SEP was considerably greater than that of lombricine when these compounds were isolated from an extract of whole worm (Table 1, Expt. 1). A similar relationship was found to exist between the specific radioactivities of these compounds whether isolated from the muscle or from the gut (Table 1, Expt. 2). In addition, the specific radioactivity of each compound was higher in the gut than in the muscle, where the bulk of the radioactivity of lombricine was found to reside in the guanidinoethanol portion of the molecule.

Labelled ethanolamine was detected in the muscle of a worm killed 4 days after injection (Table 2, Expt. 2) and a considerable amount of radioactivity was also found in glutamic acid and glutamine. The

Table 1. *Incorporation of radioactivity from* [1:2-$^{14}C_2$]-*ethanolamine into serine ethanolamine phosphodiester and lombricine of the earthworm*

The amount administered was 3·7 μmoles of [1:2-$^{14}C_2$]-ethanolamine, containing $7·5 \times 10^6$ counts/min./g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./μmole)		
	Expt. 1 (3 days)	Expt. 2 (4 days)	
	Whole worm	Muscle	Gut
SEP	231 000	67 900	126 000
Lombricine (total)	2 650	1 080	2 580
Lombricine (serine moiety)	—	60 (6%)	—
Lombricine (guanidinoethanol moiety)	—	1 020 (94%)	—

Table 2. *Incorporation of radioactivity from* [1:2-$^{14}C_2$]*ethanolamine into the free amino acids of the earthworm*

The amount administered was 3·7 μmoles of [1:2-$^{14}C_2$]-ethanolamine, containing $7·5 \times 10^6$ counts/min./g. body wt.

	Specific radioactivity (counts/min./μmole)	
	Expt. 1 (3 days) Whole worm	Expt. 2 (4 days) Muscle
Glutamic acid	156 000	38 900
Glutamine	72 000	48 500
Aspartic acid	21 800	7 400
Alanine	18 900	4 100
Glycine	5 900	3 400
Threonine	4 800	6 300
Serine	3 800	3 600
Ethanolamine	—	409 000

Table 3. *Incorporation of radioactivity from* DL-[3-^{14}C]*serine into serine ethanolamine phosphodiester and lombricine of the muscle and gut of the earthworm*

The amount administered was 2 μmoles of DL-[3-^{14}C]serine (10^7 counts/min.)/g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./μmole)		
	Expt. 1 (2 days)	Expt. 2 (8 days)	Expt. 3 (9 days)
Muscle			
SEP	31 500	41 700	70 200
Lombricine (total)	900	6 270	15 700
Lombricine (serine moiety)	680 (76%)	4 450 (71%)	9 340 (60%)
Lombricine (guanidinoethanol moiety)	225 (24%)	1 820 (29%)	6 360 (40%)
Gut			
SEP	100 000	222 000	422 000
Lombricine	1 730	9 900	27 500
	Specific radioactivity of SEP/specific radioactivity of lombricine		
	Expt. 1 (2 days)	Expt. 2 (8 days)	Expt. 3 (9 days)
Muscle	35	7	5
Gut	58	22	15

Table 4. *Incorporation of radioactivity from* DL-[3-^{14}C]*serine into the free amino acids of the muscle of the earthworm*

The amount administered was 2 μmoles of DL-[3-^{14}C]-serine, containing 10^7 counts/min./g. body wt.

	Specific radioactivity (counts/min./μmole)	
	Expt. 1 (8 days)	Expt. 2 (9 days)
Glutamic acid	71 100	173 000
Glutamine	62 700	243 000
Aspartic acid	27 800	63 900
Alanine	32 800	57 400
Glycine	10 500	28 700
Threonine	6 700	28 400
Serine	12 800	57 400

other amino acids investigated showed a much lower level of incorporation, which, with serine, is indicative of little tendency of ethanolamine to act as a precursor of this amino acid. A similar conclusion may be drawn from the fact that only a small proportion of the total radioactivity of muscle lombricine could be found in the serine part of the molecule (Table 1, Expt. 2).

DL-[3-^{14}C]*Serine*. After the administration of DL-[3-^{14}C]serine there was a progressive increase in the incorporation of radioactivity into SEP and lombricine in both the muscle and gut (Table 3). The specific radioactivity of SEP at each time interval was considerably greater than that of lombricine for each tissue and the specific radioactivities of both SEP and lombricine in gut were greater than those of muscle. With time the specific radioactivity of SEP more nearly approached that of lombricine for both tissues, as evidenced by the progressive fall in the SEP/lombricine ratios.

The greater part of the radioactivity of muscle lombricine was in the serine portion of the molecule but, even after only 2 days, 24% of the radioactivity was in the guanidinoethanol portion, presumably as a result of decarboxylation of serine to form ethanolamine.

The specific radioactivities of the free amino acids of muscle observed after the administration of [^{14}C]serine bear a relation to each other similar to that found after the administration of [^{14}C]ethanolamine (Table 2). Thus there was considerable incorporation into glutamic acid and glutamine, but less incorporation into aspartic acid, alanine, glycine and threonine. However, whereas the specific radioactivities of the well-labelled glutamic acid and glutamine were less than that of muscle SEP when [^{14}C]ethanolamine was the source of the radioactivity (cf. Tables 1 and 2), this was not so when the radioactivity was derived from [^{14}C]serine (cf Tables 3 and 4).

The results of a typical experiment with [^{14}C]-serine are illustrated in Figs. 1 and 2. In both figures the lines drawn around the ninhydrin-stained areas of SEP, lombricine and free amino acids on the chromatograms (A) were superimposed on the radioautographs (B). Muscle (Fig. 1) contained considerably more lombricine than SEP but, because of the lower specific radioactivity of lombricine, the darkening of the X-ray

film in the lombricine area was much less. In gut (Fig. 2) the amounts of SEP and lombricine were similar and approximately equal to the amount of SEP in muscle. However, the darkening of X-ray film caused by SEP was much greater for gut (Fig. 2) than for muscle (Fig. 1), because of the greater specific radioactivity of gut SEP. The labelling of lombricine in gut is not evident in the reproduction of the radioautograph shown in Fig. 2, but in the original X-ray film a definite darkening could be seen in this area.

The intensity of the spots corresponding to the free amino acids in both the chromatograms and radioautographs was as anticipated, having regard to the concentrations and specific radioactivities of those substances.

L-[^{3}H]*Serine and* D-[^{3}H]*serine.* 2 and 8 days after the administration of either L-[^{3}H]serine or D-[^{3}H]-serine, radioactivity was incorporated into both SEP and lombricine in the muscle and gut (Table 5). As in the previous experiments, the specific radioactivities of both substances were greater in gut than in muscle, and for both tissues the specific radioactivity of SEP was greater than that of

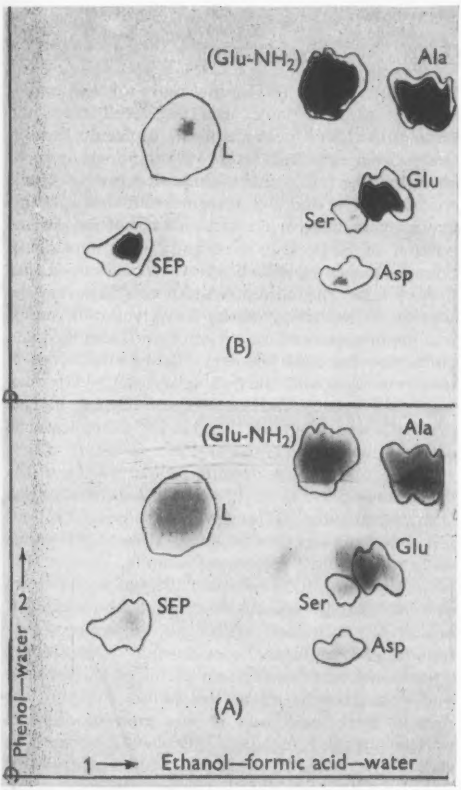


Fig. 1. (A) Chromatogram of extract of earthworm muscle prepared 8 days after the administration of 2 μmoles of DL-[3-^{14}C]serine containing 10^7 counts/min./g. body wt. SEP, serine ethanolamine phosphodiester; L, lombricine; (Glu-NH$_2$), glutamine; Ala, alanine; Glu, glutamic acid; Ser, serine; Asp, aspartic acid. (B) Radioautograph of chromatogram shown in (A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

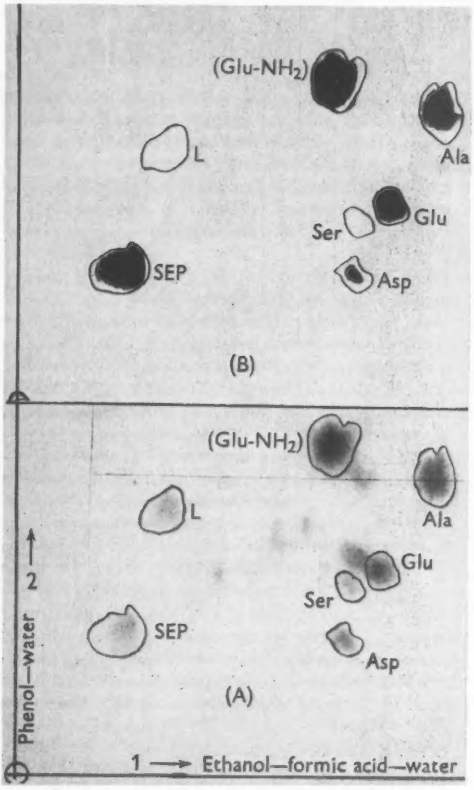


Fig. 2. (A) Chromatogram of extract of earthworm gut prepared 8 days after the administration of 2 μmoles of DL-[3-^{14}C]serine containing 10^7 counts/min./g. body wt. Abbreviations are as given in Fig. 1. (B) Radioautograph of chromatogram shown in (A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

Table 5. *Incorporation of radioactivity from either* L-[^{3}H]*serine or* D-[^{3}H]*serine into the serine ethanolamine phosphodiester and lombricine of the muscle and gut of the earthworm*

The amount administered was 10 μmoles of L-[^{3}H]serine or D-[^{3}H]serine, containing 4×10^7 counts/min./g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./μmole)										
	2 days					8 days					
	L-[^{3}H]Serine			D-[^{3}H]Serine		L-[^{3}H]Serine			D-[^{3}H]Serine		
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 8	Expt. 9	Expt. 10	Expt. 11
Muscle											
SEP	41 300	35 200	9 190	8 800	24 900	13 400	30 900	52 600	70 000	33 200	23 900
Lombricine (total)	1 670	1 270	1 300	220	550	1 560	1 360	6 500	5 260	2 910	2 600
Lombricine (serine moiety)	575 (34%)	430 (33%)	300 (23%)	140 (64%)	220 (40%)	680 (44%)	460 (34%)	—	2 750 (52%)	1 140 (39%)	—
Lombricine (guanidinoethanol moiety)	1 095 (66%)	840 (67%)	1 000 (77%)	80 (36%)	330 (60%)	880 (56%)	900 (66%)	—	2 510 (48%)	1 770 (61%)	—
Gut											
SEP	101 000	70 000	181 000	28 600	199 000	80 600	30 300	64 000	191 000	122 000	22 100
Lombricine (total)	5 470	2 910	9 230	860	8 660	—	—	15 000	7 670	14 700	8 000
Lombricine (serine moiety)	920 (17%)	640 (22%)	2 310 (25%)	315 (37%)	1 660 (19%)	—	—	—	3 900 (51%)	2 800 (24%)	—
Lombricine (guanidinoethanol moiety)	4 550 (83%)	2 270 (78%)	6 920 (75%)	545 (63%)	7 000 (81%)	—	—	—	3 770 (49%)	11 900 (76%)	—

Table 6. *Incorporation of radioactivity from either* L-[^{3}H]*serine or* D-[^{3}H]*serine into the free amino acids of the muscle of the earthworm*

The amount administered was 10 μmoles of L-[^{3}H]serine or D-[^{3}H]serine, containing 4×10^7 counts/min./g. body wt. The worms were killed after 2 days.

	Specific radioactivity (counts/min./μmole)	
	Expt. 1. L-[^{3}H]Serine	Expt. 2. D-[^{3}H]Serine
Glutamic acid	35 900	44 700
Glutamine	53 400	27 000
Aspartic acid	27 900	33 100
Alanine	22 400	15 900
Glycine	42 600	48 600
Serine	262 000	267 000

lombricine. With [^{3}H]serine more of the radioactivity of lombricine was recovered from the guanidinoethanol portion of the molecule than in the experiments with DL-[3-^{14}C]serine. It is probable that the [^{3}H]serine is degraded into a C_2-unit containing C-1 and C-2 of serine, and that it is this C_2-unit that is incorporated into the ethanolamine portion of SEP, and eventually into lombricine. Some evidence for this lies in the observation that 2 days after the administration of [^{3}H]serine the specific radioactivity of the free glycine of muscle was of the same order as that of glutamic acid and glutamine for both isomers (Table 6). This is in sharp contrast with the finding for DL-[3-^{14}C]serine, where the specific radioactivity of the free glycine of muscle was much less than that of either glutamic acid or glutamine (Table 4).

The figures for the specific radioactivities of the other free amino acids of muscle 2 days after the administration of [^{3}H]serine show no consistent differences between the labelling from L-[^{3}H]serine and that from D-[^{3}H]serine (Table 6).

L-[amidino-^{14}C]*Arginine*. Experiments were carried out to determine the origin of the amidine-C atom of lombricine. After the injection of L-[*amidino*-^{14}C]arginine, considerable radioactivity was recovered from the lombricine of both muscle and gut. Almost all of the radioactivity of the muscle lombricine was in the guanidinoethanol portion of the molecule (Table 7). Negligible radioactivity was found in SEP of both muscle and gut, and in the serine portion of the muscle lombricine; ornithine and glutamic acid also contained negligible radioactivity.

DISCUSSION

On the basis of the structure of lombricine it would be anticipated that administered serine and ethanolamine would both be incorporated, bio-

Table 7. *Incorporation of radioactivity from* L-[amidino-^{14}C]*arginine into the serine ethanolamine phosphodiester, lombricine and certain free amino acids of the earthworm*

The amount administered was 20 μmoles of L-[*amidino*-^{14}C]arginine, containing 10^6 counts/min./g. body wt. The worms were killed after 2 days. Figures in parentheses represent the percentage of the counts in the guanidinoethanol portion of the lombricine molecule. 'Negligible' indicates that the net counting rate was less than one-half of the background counting rate.

	Expt. 1. Counts/min./g. of worm		Expt. 2.* Specific radioactivity (counts/min./μmole)	
	Muscle	Gut	Muscle	Gut
SEP	Negligible	Negligible	Negligible	Negligible
Lombricine (total)	540	120	329	382
Lombricine (serine moiety)	Negligible	—	Negligible	Negligible
Lombricine (guanidinoethanol moiety)	525 (97%)	—	318 (97%)	348 (91%)
Ornithine	Negligible	—	Negligible	—
Glutamic acid	—	—	Negligible	—
Arginine	12 400	—	38 700	—

* Twice the amount of [^{14}C]arginine was given.

synthetically, into the molecule. Further, because of the well-known inter-relationship existing between serine and ethanolamine, it would be expected that some of the radioactivity in [3-^{14}C]-serine would appear in the guanidinoethanol portion of the molecule. The results of the present experiments confirm these expectations.

In all experiments carried out with [^{14}C]- and [^{3}H]-serine and ethanolamine, the specific radioactivity of SEP was considerably greater than that of lombricine for both gut and muscle tissue. This finding is similar to that obtained with inorganic ^{32}P and reported elsewhere (Gaffney *et al.* 1960). It is consistent with the view expressed (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959) that SEP is a precursor of lombricine and not a degradation product of that substance. The report (Ennor *et al.* 1960) that the serine moiety of SEP, like that of lombricine, has the unusual D-configuration, is also consistent with this view. In addition, the present experiments make it clear that SEP is built up from small molecules, a finding at variance with the conclusion of Ayengar & Roberts (1957) for the SEP of turtles. However, in contrast with earthworm SEP, the SEP of turtles contains serine with the L-configuration (Roberts & Lowe, 1954).

These experiments confirm the previously reported slow turnover rate of lombricine in the earthworm (Rossiter *et al.* 1960; Gaffney *et al.* 1960). In each instance the amount of radioactivity administered was adjusted to the body weight of the worm. The results clearly show that with all of the precursors studied considerable radioactivity was absorbed from the gut, although some activity (about 14% in the control experiment) was lost with the large quantity of earth excreted by the worm during the course of the experiment. Further, it was noted that some worms visibly ejected a portion of the administered material through the outer surface of the skin. The variable nature of the amount of radioactive precursors absorbed, or retained when once absorbed, thus did not permit of the plotting of smooth 'precursor–product' curves showing changes in specific radioactivity with time.

Since both L- and D-[^{3}H]serine are incorporated into SEP and lombricine of earthworms (as also is DL-[^{14}C]serine), it might be anticipated that the D-enantiomorph is incorporated directly and that the L-isomer is changed into the D-form before or at the moment of incorporation into SEP. However, the present experiments provide no data on this point, or on the mechanism of the conversion of L-serine into D-serine.

In the experiments with L-[*amidino*-^{14}C]arginine there was negligible radioactivity in SEP and considerable radioactivity in lombricine. Degradation experiments have shown that this radioactivity is restricted to the guanidinoethanol portion of the molecule and are strongly suggestive of the possibility that the amidino group of lombricine is derived from that of arginine (Scheme I).

The site of lombricine formation cannot be determined from these experiments, but with each of the isotopically labelled compounds used ([1:2-$^{14}C_2$]-ethanolamine, DL-[3-^{14}C]serine, L-[^{3}H]serine and D-[^{3}H]serine) the specific radioactivity of SEP was greater in the gut than in the muscle. The indication thus is that SEP is formed in one or more of the components of the gut; a similar conclusion has been reached as a result of experiments with inorganic ^{32}P (Gaffney *et al.* 1960). The site of the final reaction of SEP to give lombricine has yet to be determined, but the present results suggest that a transamidinase reaction is involved.

SUMMARY

1. When [1:2-$^{14}C_2$]ethanolamine, DL-[3-^{14}C]-serine, L-[^{3}H]serine or D-[^{3}H]serine was administered orally to earthworms, serine ethanolamine phosphodiester and lombricine were found to be radioactively labelled.

2. For each of the precursors the specific radioactivity of serine ethanolamine phosphodiester was greater than that of lombricine in both muscle and gut tissue.

3. The specific radioactivity of serine ethanolamine phosphodiester and lombricine was greater in gut than in muscle.

4. With [1:2-$^{14}C_2$]ethanolamine most of the radioactivity of muscle lombricine was in the guanidinoethanol portion of the molecule, whereas with DL-[3-^{14}C]serine most of the radioactivity was in the serine portion.

5. When L-[*amidino*-^{14}C]arginine was given, there was negligible radioactivity in serine ethanolamine phosphodiester and considerable radioactivity in lombricine, almost all of which was in the guanidinoethanol portion of the molecule.

6. These findings suggest that serine ethanolamine phosphodiester is the precursor of lombricine in the earthworm and that the amidino group is derived from arginine by the transamidinase-catalysed transfer of the amidino group to serine ethanolamine phosphodiester.

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REFERENCES

Ayengar, P. & Roberts, E. (1957). *Fed. Proc.* **16**, 147.
Beatty, I. M. & Magrath, D. I. (1959). *Nature, Lond.*, **183**, 591.
Beatty, I. M., Magrath, D. I. & Ennor, A. H. (1959). *Nature, Lond.*, **183**, 591.
Connell, G. E., Dixon, G. H. & Hanes, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 416.
Ennor, A. H. & Morrison, J. F. (1958). *Physiol. Rev.* **38**, 631.
Ennor, A. H., Rosenberg, H., Rossiter, R. J., Beatty, I. M. & Gaffney, T. J. (1960). *Biochem. J.* **75**, 179.
Jones, E. E. & Lipkin, D. (1956). *J. Amer. chem. Soc.* **78**, 2408.
Gaffney, T. J., Rossiter, R. J., Rosenberg, H. & Ennor, A. H. (1960). *Biochem. biophys. Acta*, (in the Press).
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Passmann, J. M., Radin, N. S. & Cooper, J. A. D. (1956). *Analyt. Chem.* **28**, 484.
Roberts, E. & Lowe, I. P. (1954). *J. biol. Chem.* **211**, 1.
Rosenberg, H. (1959). *Biochem. J.* **72**, 582.
Rosenberg, H. & Ennor, A. H. (1959). *Biochem. J.* **73**, 521.
Rossiter, R. J., Gaffney, T. J., Rosenberg, H. & Ennor, A. H. (1960). *Nature, Lond.*, **185**, 383.
Stetten, De W. & Bloom, B. (1956). *J. biol. Chem.* **220**, 723.
Van Thoai, N. & Robin, Y. (1954). *Biochim. biophys. Acta*, **14**, 76.
Vaughan, M., Steinberg, D. & Logan, J. (1957). *Science*, **126**, 446.
Wilzbach, K. E. (1957). *J. Amer. chem. Soc.* **79**, 1013.

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Structure of the Ribitol Teichoic Acid from the Walls of *Bacillus subtilis*

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Some micro-organisms contain in their walls a considerable amount of a polymer belonging to the group of phosphoric esters known as teichoic acids (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley, 1959; Armstrong *et al.* 1959*b*). These compounds are polymers of glycerophosphate or ribitol phosphate in which adjacent polyol residues are joined together through phosphodiester linkages. In addition, the glycerol teichoic acids contain alanine in ester linkage with a hydroxyl on the glycerol, whereas ribitol teichoic acids contain both alanine ester linkages and sugars or *N*-acylamino sugars. Hydrolysis of ribitol teichoic acids with acid, or with alkali followed by phosphatase treatment, suggests that the sugars are attached glycosidically to hydroxyl groups in the ribitol residues.

In the present work the structure of the ribitol teichoic acid from the walls of *Bacillus subtilis* is discussed. A preliminary account of some of these results has been given elsewhere (Armstrong, Baddiley & Buchanan, 1959*a*).

* Salters' Student.

EXPERIMENTAL

Materials. Prostate phosphatase was kindly supplied by Dr G. Schmidt and β-glucosidase by Dr W. J. Whelan. D-Alanine and D-amino acid oxidase were purchased from

L. Light and Co. Ltd., Colnbrook, Bucks. *B. subtilis* was grown from a culture provided by Dr M. R. J. Salton.

Paper chromatography

Paper chromatography was carried out on Whatman no. 1 or no. 4 paper which had been washed with 2N-acetic acid and then water. The following solvent systems were used: A, propanol–ammonia (sp.gr. 0·88)–water (6:3:1) (Hanes & Isherwood, 1949); B, butanol–ethanol–water–ammonia (sp.gr. 0·88) (40:10:49:1) (organic phase) (Foster, Horton & Stacey, 1957); C, butanol–pyridine–water (6:4:3) (Jeanes, Wise & Dimler, 1951); D, phenol–water (9:1). The products were detected by E, the improved periodate–Schiff reagent for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956*b*); F, molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949); G, aniline phthalate reagent for reducing sugars (Partridge, 1949); H, ninhydrin reagent for amino acids (Consden & Gordon, 1948).

Isolation of teichoic acid from Bacillus subtilis *cells*

B. subtilis was grown in bottles (10 l.) with aeration in a peptone (2%)–sodium chloride (0·5%) liquid medium. Growth from an inoculum (250 ml.) of fresh cells was allowed to take place for 48 hr. at 22°. The cells were harvested in a Sharples centrifuge and washed with 0·95% sodium chloride solution at 0°. They were successively treated in a blender (30 sec.) with acetone, ethanol and ether (15 vol. of each) at room temperature.

Short cold extraction. Fat-free cells (36 g.) were extracted three times with 150 ml. portions of ice-cold 10% trichloroacetic acid in a blender for 1 min. After centrifuging, the combined supernatant solutions were filtered through Supercel silica, 2 vol. of ethanol and 2 vol. of acetone were added and the solution was kept for 24 hr. at 0°. The material collected by centrifuging was reprecipitated from ice-cold 10% trichloroacetic acid (100 ml.) by the addition of 2 vol. of ethanol, kept for 18 hr. at 0°, then centrifuged and washed with ethanol and ether. The teichoic acid (200 mg.) contained small amounts of nucleic acid and polysaccharide.

Prolonged cold extraction. The cell debris from the short extraction was treated in a blender (3 min.) with ice-cold 10% trichloroacetic acid (250 ml.), stirred for 18 hr. at 0°, then treated further (1 min.) in the blender. The supernatant solution, after centrifuging of the debris, was filtered through Supercel silica and mixed with 3 vol. of ethanol. After 24 hr. at 0° the material was collected by centrifuging and reprecipitated from 10% trichloroacetic acid (50 ml.) with ethanol (2 vol.). After 18 hr. at 0° the precipitate was removed by centrifuging and dried with acetone and ether. The teichoic acid (1·23 g.) contained 4% of nucleic acid and traces of a glycerophosphate derivative which is probably a glycerol teichoic acid. The addition of a further ½ vol. of ethanol to the supernatant and then allowing it to stand for 24 hr. at 0° yielded a precipitate which was collected by centrifuging and dried with acetone and ether. This teichoic acid (311 mg.) contained 5% of nucleic acid and traces of the glycerophosphate derivative and polysaccharide.

Extraction at 50°. The cell residue from the short cold extraction of fat-free whole cells (15 g.) was stirred with 5% trichloroacetic acid (300 ml.) for 15 min. at 50°. After centrifuging, the supernatant solution was filtered through Supercel silica and the trichloroacetic acid extracted with 6 × 150 ml. of ether. The resulting precipitate was removed by centrifuging, the pH of the supernatant was adjusted to 6·3 with ammonia and the volume reduced to 15 ml. by evaporation at 25° under reduced pressure. The solution was cooled and 5 vol. of ethanol were added. After 48 hr. the insoluble material was centrifuged and reprecipitated from 50 ml. of 10% trichloroacetic acid by the addition of 2 vol. of ethanol. After 24 hr. at 0° the precipitate was collected by centrifuging and dried with acetone and ether. The teichoic acid (0·65 g.) contained 5–10% of nucleic acid and glycerol teichoic acid in larger amounts than that present in material obtained from cold extractions.

Isolation of teichoic acid from Bacillus subtilis *walls*

The organism was grown for 18 hr. at 30° as described previously. The cells (20 g.) were harvested in a Sharples centrifuge, washed with 0·95% sodium chloride solution and water and then suspended in water (175 ml.) at 0°. Portions of the suspension (10 ml.) were mixed with portions (10 ml.) of Ballotini beads and shaken in a Mickle disintegrator for 20 min. at 0°. The beads were removed by filtration through a no. 1 sintered-glass funnel and the filtrate was centrifuged at 15 000 rev./min. for 30 min. The top layer of cell contents, the middle layer of walls and the dense bottom layer of whole cells were separated and the wall fraction was treated in a homogenizer with 0·1M-phosphate buffer, pH 7·0. The suspension was centrifuged at 12 000 rev./min. for 20 min., and again the whole cells were removed. This process was repeated 14 times and was followed by ten washes with water and centrifuging at 15 000 rev./min. for 30 min. The preparation was examined under the electron microscope for whole cells and cell contents. The walls (30 mg.) (Found: P, 4·1%) were freeze-dried and samples (2·0 mg.) hydrolysed with 2N-hydrochloric acid for 3 hr. at 100°. After removal of hydrochloric acid by evaporation the residues were chromatographed in solvent A. The papers were examined under ultraviolet light and sprayed with reagents E and F (see below).

B. subtilis walls (120 mg.) were stirred with 10% trichloroacetic acid (5 ml.) for 24 hr. at 0°. The walls were centrifuged and extracted twice with portions (5 ml.) of 10% trichloroacetic acid. The supernatant solutions from each extraction were mixed with ethanol (5 vol.) and kept for 24 hr. at 0°. The precipitates were collected by centrifuging and dried with ethanol and ether. The combined precipitates (40 mg.) were dissolved in 10% trichloroacetic acid (2·0 ml.) and fractionally precipitated with ethanol (1·5, 2·5 and 4·5 vol.). The first two precipitates (36 mg.) contained pure teichoic acid. The third contained teichoic acid contaminated with polysaccharide.

The wall residue was suspended in a little water and the trichloroacetic acid was removed by six extractions with ether (½ vol.). The residue was freeze-dried (74 mg.) (Found: P, 1·83%).

Teichoic acid

Acid hydrolysis. Teichoic acid (2·0 mg.), isolated from walls, was hydrolysed in 2N-hydrochloric acid in a sealed tube for 3 hr. at 100°. After evaporation the samples were examined by paper chromatography in solvents A, B, C and D.

Products are given in Table 1 and R_F values in Table 2.

Analysis. Teichoic acid (1·0 mg.), isolated from walls, was hydrolysed in 2N-hydrochloric acid for 9 hr. at 100°. The hydrolysate was neutralized to pH 7·0 with sodium hydroxide and the volume adjusted to 5·0 ml. Phosphorus (Chen, Toribara & Warner, 1956), glucose (Park & Johnson, 1949) and alanine (Rosen, 1957) were determined. The glucose value was corrected for destruction (12%) by acid during hydrolysis. This correction was estimated by heating glucose and alanine together under the conditions of the hydrolysis and determining the amount of glucose destroyed. (Found: ratio P:glucose:alanine, 1·0:0·93:0·46.)

Alkali hydrolysis and enzymic dephosphorylation. Teichoic acid (650 mg.) isolated from whole cells by extraction at 50° (see above) was dissolved in N-sodium hydroxide (7·0 ml.) and the resulting solution heated for 3 hr. at 100°. A slight red-brown precipitate (probably ferric hydroxide) was removed by centrifuging and washed with water. The supernatant and washings were adjusted to 25 ml. with water and passed through a Dowex-50 (NH_4^+ form) (25 ml.) column and the column was washed with water (500 ml.). The eluate was decreased to 25 ml. by evaporation at 30° under reduced pressure. A sample (0·1 ml.) was removed and examined in solvents A and B with reagents E, F, G and H. The results are shown in Table 1.

Table 1. *Hydrolysis products of teichoic acid*

Teichoic acid was hydrolysed for 3 hr. in HCl (2N) or NaOH (N) at 100°. The products were identified by chromatography (see text).

	Acid hydrolysis	Alkali hydrolysis
1:4-Anhydroribitol	+	−
Ribitol	+	+*
Glucose	+	−
Alanine	+	+
Glucosylribitol	+	+
Ribitol phosphates	+	+*
Glucosylribitol phosphates	+	+
1:4-Anhydroribitol 5-phosphate	+	−
Inorganic phosphate	+	−

* Traces only.

Table 2. *Paper chromatography of acid hydrolysis products of teichoic acid*

Solvents used were propanol–ammonia (A) and butanol–ethanol–water–ammonia (B).

	R_F	R_{ribitol}
	A	B
Anhydroribitol	0·75	1·38
Glycerol	0·75	1·47
Ribitol	0·65	1·00
Glucose	0·61	0·65
Alanine	0·63	0·57
Glucosylribitol	0·55	0·55
Ribitol 1-phosphate	0·30	·
Ribitol 2 (and 3)-phosphate	0·33	·
Anhydroribitol 5-phosphate	0·29	·
Glucosylribitol 1 (and 2)-phosphate	0·28	·
Ribitol diphosphate	0·12	·
Glucosylribitol diphosphate	0·12	·
Glycerol diphosphate	0·12	·

The solution was neutralized to pH 5·5 with acetic acid, 0·4M-ammonium acetate buffer (pH 5·5, 15 ml.) and prostate-phosphatase solution (1·5 ml.) were added and the volume was adjusted to 50 ml. with water. The solution was incubated at 37° under toluene, samples being removed at intervals and analysed for inorganic phosphate and total phosphorus (Fiske & Subbarow, 1925). After 3 days 91% of the phosphorus had been released as inorganic phosphate. A further 0·5 ml. of prostate-phosphatase solution was added and the incubation was continued for a further 17 hr., after which time 99·3% of the phosphorus had been released as inorganic phosphate. The volume was adjusted to 200 ml. with water and the solution was passed quickly through a Dowex-50 (H^+ form) (50 ml.) resin column cooled to 0–5°. The eluate and 250 ml. of column washings were passed directly into a flask containing 30 ml. of Dowex-2 (CO_3^{2-} form) resin. The contents of the flask were poured on to a Dowex-2 (CO_3^{2-} form) (10 ml.) column and the column was washed with water (500 ml.). The eluate was evaporated at 30–35° under reduced pressure to a small volume and then freeze-dried. The dry white solid (386 mg.) was examined in solvents A and B and contained only glycerol, ribitol and glucosylribitol.

The solid (200 mg.) was suspended in hot ethanol and water was added carefully until the solid just dissolved. The solution was kept at room temperature overnight. The rosettes of white needles were filtered off, washed with ethanol and ether, dried and recrystallized from hot aqueous ethanol. The glucosylribitol (157 mg.) had m.p. 137–138°; $[\alpha]_D^{21} - 28{\cdot}6°$ in water (c, 1·0). (Found: C, 41·9; H, 7·3. $C_{11}H_{22}O_{10}$ requires C, 42·0; H, 7·1%). It was examined in solvents A and B and contained no glycerol or ribitol.

Potentiometric titration. Teichoic acid (8·0 mg.) prepared from walls was dissolved in aqueous 2N-ammonia (2·0 ml.) and the solution kept for 5 hr. at room temperature. The excess of ammonia was evaporated, water (5·0 ml.) was added and the solution was passed through a Dowex-50 (H^+ form) (3·0 ml.) resin column to remove alanine, alanine amide and NH_4^+ ions. The column was washed with water (10 ml.) and the eluate and washings were adjusted to 20 ml. with water. The teichoic acid in this solution was titrated against 0·01N-sodium hydroxide, the change of pH being followed on a Pye pH meter. A plot of $\Delta pH/\Delta V$ against V, where V is the volume of alkali used, gave two peaks, indicating the points of neutralization of the primary and secondary acidic groups. The ratio of primary to secondary groups was 8·9:1.

Periodate oxidation. The alanine residues in teichoic acid (7·0 mg.) from walls were removed by treatment with ammonia as in the previous experiment. The eluate from the Dowex-50 (H^+ form) (3·0 ml.) resin column was neutralized to pH 6·5 with sodium hydroxide, 0·3M-sodium metaperiodate solution (0·4 ml.) was added, the volume was adjusted to 10 ml. with water and the solution was kept at room temperature in the dark. A control experiment was carried out without added periodate. Samples (2·0 ml.) were used to determine the periodate used and the formaldehyde formed (Hough, Powell & Woods, 1956); samples (0·1 ml.) were also used for total phosphorus determination (Chen *et al.* 1956). After 60 hr. 51 μmoles of periodate had been used. 1·96 μmoles of formaldehyde were formed and 17·2 μmoles of phosphorus were present. The ratio of periodate uptake to formaldehyde formed was 26:1; P:formaldehyde, 8·8:1; periodate

uptake: P, 2·97:1. Ratios required for structure (VII) are 28, 9 and 3·1 to 1 respectively.

Action of prostate phosphatase. Teichoic acid (5·2 mg.) from walls was dissolved in water (7·0 ml.). 0·4 M-Ammonium acetate buffer (2·0 ml.), pH 5·5, and prostate phosphatase (0·2 ml.) were added, and the volume was adjusted to 10 ml. with water. The solution was incubated under toluene at 37° and at intervals samples (0·2 ml.) were removed for total phosphorus determination and larger samples (2·0 ml.) for inorganic phosphate determination (Chen *et al.* 1956). After 90 hr. the ratio total P:inorganic P was 27:1. A further quantity (0·1 ml.) of phosphatase was added and incubation continued for 66 hr. The ratio total P:inorganic P decreased to 21:1 (the ratio expected from structure (VII) is 9:1).

Isolation of alanine. Teichoic acid (600 mg.), prepared from whole cells by prolonged cold extraction was hydrolysed with N-sodium hydroxide (6·0 ml.) at 100° for 3 hr. A small amount of ferric hydroxide was removed by centrifuging and washed with water. The supernatant and washings were adjusted to pH 6·2 with acetic acid and a solution of barium hydroxide (350 mg.) in water (2·0 ml.) was added. Ethanol (400 vol.) was added with stirring and the mixture was kept overnight at 0°. The precipitated barium salts (0·72 g.) were collected by centrifuging and washed twice with ethanol (250 ml.) and ether and dried over phosphoric oxide. Chromatography in solvent A (spray reagents E, F and H) indicated that these barium salts contained the normal phosphates produced by alkali hydrolysis of teichoic acid together with a small amount of alanine.

The supernatant and ethanol washings, containing the bulk of the alanine, were diluted with water (65 ml.) and passed through a Dowex-50 (H^+ form) (35 ml.) resin column. The column was washed with water (500 ml.); no amino compounds were detected in the eluate. The alanine was eluted from the column with 2 N-hydrochloric acid, 5 ml. fractions being collected. Appropriate fractions were evaporated to dryness under reduced pressure at 30° and kept over potassium hydroxide. The dry solid was dissolved in water (1 l.), the pH was adjusted to 7 with sodium hydroxide and the solution was passed through a Dowex-2 (OH^- form) (50 ml.) resin column. The column was washed with water (1 l.) and the eluate and washings were checked with ninhydrin for the absence of alanine. Alanine was eluted (5 ml. fractions) with N-hydrochloric acid and appropriate fractions were combined and evaporated to dryness under reduced pressure at 30°. Repeated addition of water (0·5 ml.) and evaporation to dryness over potassium hydroxide failed to remove traces of hydrochloric acid. The alanine hydrochloride (89 mg.) was slightly yellow due to impurities from the resin. Chromatography in solvents A and D and butanol–water and spraying with reagent H failed to detect impurities.

Configuration of alanine. The alanine hydrochloride (6·0 mg.) was dissolved in water (2·0 ml.) and samples (0·2 ml.) were diluted to 10 ml. with 0·05 M-pyrophosphate buffer, pH 8·2. The concentration of alanine in these samples was determined colorimetrically with ninhydrin (Rosen, 1957). Standard solutions containing 20 μmoles of D- and L-alanine/ml. were prepared in 0·05 M-pyrophosphate buffer, pH 8·2. Enzymic oxidation was measured in a Warburg apparatus on solutions containing 8 μmoles of amino acid, 5 μg. of catalase, 20 mg. of D-amino acid oxidase and buffer to a final volume of 3·2 ml. The centre well contained 20% potassium hydroxide (0·2 ml.) and the reaction was carried out in air at 37°. The alanine from teichoic acid consumed 0·49 mole of oxygen/mole and authentic D-alanine consumed 0·5 mole of oxygen/mole. No oxygen was consumed by authentic L-alanine.

Glucosylribitol

Acid hydrolysis. The compound (1·0 mg. samples) was hydrolysed in 2 N-hydrochloric acid for 3, 12 and 24 hr. at 100°. The acid was removed by evaporation and the products were examined by paper chromatography in solvents A, B and C. After 3 hr. the compound was partially hydrolysed to ribitol, anhydroribitol and glucose; after 12 hr. hydrolysis was complete and the products were ribitol, anhydroribitol and glucose; after 24 hr. the products were anhydroribitol and glucose.

Periodate oxidation and borohydride reduction. Glucosylribitol (1·0 mg.) was dissolved in 1% sodium metaperiodate solution (0·4 ml.). It was left for 18 hr. at room temperature, sodium borohydride (2·0 mg.) was added and the solution was kept for a further 24 hr. 2 N-Hydrochloric acid (0·2 ml.) was added and the solution was heated for 30 min. at 100°. After evaporation under reduced pressure the product was examined in ethyl acetate–pyridine–water (7:2:1) (Viscontini, Hoch & Karrer, 1955). The only observed product rapidly gave a purple colour with reagent E and had R_F 0·43 (glycerol had R_F 0·43, ethylene glycol had R_F 0·55).

Periodate oxidation. Glucosylribitol (15·8 mg.) was dissolved in water (25 ml.), 0·3 M-sodium metaperiodate (2·0 ml.) was added and the volume was adjusted to 50 ml. with water. A control experiment containing no glucosylribitol was also carried out. The solutions were kept at room temperature in the dark. Samples (5 ml.) were used for the determination of periodate consumed and formic acid produced, and other samples (2 ml.) for formaldehyde determination by the method of Hough *et al.* (1956). The formic acid was titrated with 0·01 N-sodium hydroxide solution to pH 6·25. After 18 hr. glucosylribitol had consumed 3·93 mol.prop. of periodate and produced 0·96 mol.prop. of formaldehyde and 1·0 mol.prop. of formic acid. After 36 hr. 1·2 mol.prop. of formic acid had been produced but the periodate uptake and formaldebyde production were unchanged. To samples (5 ml.) of the glucosylribitol solution and to the control was added ethylene glycol (2 ml.). After 15 min. at room temperature in the dark carbon dioxide-free air was bubbled through, 0·01 N-sodium hydroxide (2 ml.) was added and the samples were kept at room temperature for 1 hr. Water (5·0 ml.) was added and formic acid production was determined by back titration with 0·01 N-oxalic acid. After this treatment 1·9 mol.prop. of formic acid was formed. Further oxidation for 24 hr. at 37° liberated 1·7 mol.prop. of formic acid.

Action of β-glucosidase. Glucosylribitol (1·0 mg.) was dissolved in water (15 μl.) and β-glucosidase (15 μl. of a 1% solution) was added. The solution was incubated under toluene for 12 hr. at 30°. The hydrolysate was examined by chromatography in solvents A and C with reagents E and G. Hydrolysis was complete, only glucose and ribitol being detected.

Oxidation and hydrolysis to glyceric acid. Glucosylribitol (32·3 mg.) and α-methyl glucoside (48 mg.) (a molar ratio of

1:2) were separately dissolved in water (10 ml.), 0·3M-sodium metaperiodate (3 ml.) was added to each and the volumes were adjusted to 25 ml. with water. The solutions were kept in the dark at room temperature for 24 hr. and then samples (2 ml.) were removed for determination of periodate consumed (α-methyl glucoside consumed 1·98 mol.prop. of periodate, glucosylribitol consumed 3·93 mol.prop.). The solutions were passed rapidly through Dowex-50 (H^+ form) (10 ml.) resin columns and the resulting acid solutions kept for 24 hr. to hydrolyse formyl esters. Dowex-2 (CO_3^{2-} form) (6 ml.) resin was added to the two solutions and the suspensions were passed through Dowex-2 (CO_3^{2-} form) (5 ml.) columns. The columns were each washed with water (250 ml.) and the neutral eluates were evaporated to 5 ml. under reduced pressure (25–30°). The optical rotations of the solutions are given in Table 3.

The solutions were each adjusted to 35 ml., benzoic acid (146 mg.) and barium hydroxide (198 mg.) were added and the mixtures shaken until the solid dissolved. Bromine (0·3 ml.) was added to the ice-cold solutions in stoppered flasks, which were kept for 60 hr. at room temperature with frequent shaking during the first hour. Air was passed through the solutions to remove excess of bromine, the volumes were decreased to 15 ml. under reduced pressure and the benzoic acid was filtered off and washed with water. The volume of the combined filtrate and washings was decreased to 8 ml., 5N-sulphuric acid (0·9 ml.) was added to each flask and the solutions were heated at 100° for 2 hr. Barium carbonate (0·6 g.) was added to each and after keeping overnight the barium sulphate was filtered off through Supercel silica and washed with water. The filtrate and washings were passed through Dowex-50 (NH_4^+ form) (5·0 ml.) resin columns and the volume was decreased to 5·0 ml. under reduced pressure. The optical rotation of these solutions was measured. To 1 ml. of each solution was added ammonium molybdate (60 mg.) and the optical rotations were measured again (see Table 3).

Chromatography of the oxidation–hydrolysis products in solvent A and detection of products with reagent E confirmed the presence of glyceric acid in both solutions.

Action of β-glucosidase on monophosphates produced by alkali hydrolysis

Teichoic acid (3·0 mg.) was hydrolysed with N-sodium hydroxide at 100° for 3 hr. The hydrolysate was passed through a Dowex-50 (NH_4^+ form) (1·0 ml.) column and chromatographed in solvent A. The monophosphate region was eluted with water (0·2 ml.) and the eluate divided into three parts, which were treated separately as follows: (*a*) water (45 μl.) and 0·2M-ammonium acetate buffer, pH 6·0 (45 μl.), were added; (*b*) 1% β-glucosidase (45 μl.) and buffer (45 μl.) were added; (*c*) 1% β-glucosidase (45 μl.), buffer (45 μl.) and sodium fluoride to a concentration of 0·012M were added. These solutions were incubated under toluene at 37° for 1 hr. then chromatographed in solvents A and C and the products detected with reagents E, F and G. The β-glucosidase hydrolysed some of the monophosphate giving glucose and an increase in the amounts of ribitol 1- and 2-phosphate. No ribitol 3-phosphate was detected. The fluoride in tube (*c*) inhibited the action of the phosphomonoesterase known to be present in the β-glucosidase preparation.

Table 3. *Optical rotations (10 cm. tube) of products during oxidation and hydrolysis of α-methyl glucoside and glucosylribitol*

For details see text.

	α° +	
	α-Methyl glucoside	Glucosylribitol
Aldehyde from periodate oxidation	0·68	0·12
Glyceric acid	0·06	0·06
Glyceric acid + molybdate	0·28	0·24

Action of phosphomonoesterase on diphosphates produced by alkali hydrolysis

Teichoic acid (2·0 mg.) isolated from whole cells by prolonged cold extraction was hydrolysed with 2N-hydrochloric acid for 3 hr. at 100°. The hydrolysate was evaporated and chromatographed in solvent A and the diphosphate region was eluted. The eluate was evaporated to dryness, the solid was dissolved in 0·4M-ammonium acetate buffer, pH 5·5 (45 μl.), prostate phosphomonoesterase (45 μl.) was added and the mixture was incubated under toluene at 37° for 3 days. The hydrolysate was chromatographed in solvent A and the products were detected with reagents E and F. The products were ribitol, glucosylribitol, inorganic phosphate and a faint trace of glycerol.

DISCUSSION

Walls prepared by a modification of Salton & Horne's (1951) method from bacteria which had been ruptured with a Mickle vibrator have been used in this work. Such preparations are free from cell contents and protoplast membrane (personal communication from Dr D. E. Hughes supported by observations in this Laboratory with various organisms). Their purity has been checked by examination with the electron microscope and by the absence of nucleic acid. Acid hydrolysates of walls contained the amino acids and sugars arising from the mucopeptide component (Salton & Ghuysen, 1957) together with products from the ribitol teichoic acid in this organism described by Armstrong *et al.* (1958). No trace of glycerol or glycerophosphates was detected, and it is concluded that the glycerol teichoic acid present in extracts of whole organism is not located in the wall. This is in agreement with observations (unpublished) on the location of teichoic acids in many other bacteria, although glycerol teichoic acids do occur in the walls of some lactobacilli and staphylococci.

A considerable amount of teichoic acid can be extracted from bacterial walls with cold 10% trichloroacetic acid, thus suggesting that it is associated ionically with the mucopeptide. The approximate amount present in the walls of *B. subtilis* has been determined from the weight of pure teichoic acid recovered from such an extract, allowance being made for that which remained

(26 %) in the extracted walls. On this basis about 59 % of the weight of the wall is teichoic acid; this value is certainly low because of losses during purification. The assumption is made that the phosphorus content of the extracted walls is a measure of the teichoic acid which has not been removed by trichloroacetic acid. This is justified, since examination of hydrolysates of extracted walls fails to reveal the presence of phosphorus derivatives other than teichoic acid.

No attempt has yet been made to study the relationship between growth conditions and teichoic acid synthesis. However, the amount of teichoic acid which can be extracted from whole bacteria does vary in different batches of organism. The composition of the medium is clearly important since, when *B. subtilis* was grown in the presence of a relatively high concentration (2 %) of glucose, direct extraction of the whole cells yielded no teichoic acid. A considerable amount of a polysaccharide was obtained from the trichloroacetic acid extract of this culture and, as microscopic examination of the organisms suggested the presence of a capsule, it is possible that this polysaccharide was of capsular origin. Polysaccharides from this organism have been described (Sharon, 1957; Sharon & Jeanloz, 1960; Janczura, Perkins & Rogers, 1960).

In earlier work on the ribitol teichoic acid from *B. subtilis* material had been prepared from extracts of whole cells. These preparations invariably contained small amounts of glycerol teichoic acid; consequently, reliable analytical information could not be obtained. Material prepared from walls did not contain glycerol derivatives, but yielded on acid hydrolysis all the products previously ascribed to the ribitol teichoic acid in this organism. No traces of amino acids other than alanine were detected and the only sugar present was glucose. Other hydrolysis products which have been identified before were inorganic phosphate, ribitol, 1:4-anhydroribitol and its 5-phosphate, and the isomeric monophosphates of ribitol. Ribitol derivatives obtained by hydrolysis are presumably racemic compounds. The relative amounts of the different products varied with the hydrolysis time as discussed previously. Under certain conditions small amounts of hitherto undetected hydrolysis products were obtained. These were incompletely separated from each other on paper chromatograms but had the R_p values expected for diphosphates. On dephosphorylation with prostate phosphatase they yielded a mixture of ribitol and the glucosylribitol described later. Small amounts of glucosylribitol monophosphates were also detected in acid hydrolysates.

The unpurified extract of walls contained in addition to teichoic acid a small amount of a compound which probably contained amino sugars. Although this compound has not been examined fully it resembles in some respects the polysaccharide from *B. subtilis* recently described by Janczura *et al.* (1960) and called by them teichuronic acid.

Analysis of the purified material from walls indicated a molar ratio (phosphorus:glucose) of almost 1:1, but the amount of alanine varied considerably according to the methods employed in extraction and purification. The ratio phosphorus:alanine reported here (1:0·46) is typical but, in view of the remarkable lability of the alanine ester linkages in this and other teichoic acids, it is probable that higher values would be obtained if milder conditions of isolation had been employed. The teichoic acid in *B. subtilis* walls is relatively difficult to extract and considerably higher values have been obtained with teichoic acids from other organisms when mild extraction and purification conditions were used.

The alanine residues in this teichoic acid are linked through their carboxyl groups to hydroxyl groups in the polymer. They are readily released in dilute alkali and react rapidly with ammonia or hydroxylamine. Moreover, the polymer shows an infrared-absorption band at 1751 cm.$^{-1}$ in potassium bromide, characteristic of a carboxylic ester. Although the rate of reaction with hydroxylamine has not been determined, the alanine residues in ribitol teichoic acid from *Lactobacillus arabinosus* react with hydroxylamine (Armstrong *et al.* 1959*b*) at a rate comparable with that for the amino acid-ester linkages in nucleic acid–amino acid compounds concerned in protein synthesis. Experiments with D-amino acid oxidase indicate that the alanine in this teichoic acid, like that from *L. arabinosus* and *Staphylococcus aureus*, has the D-configuration. The location of the alanine ester groups in the polymer is not yet known.

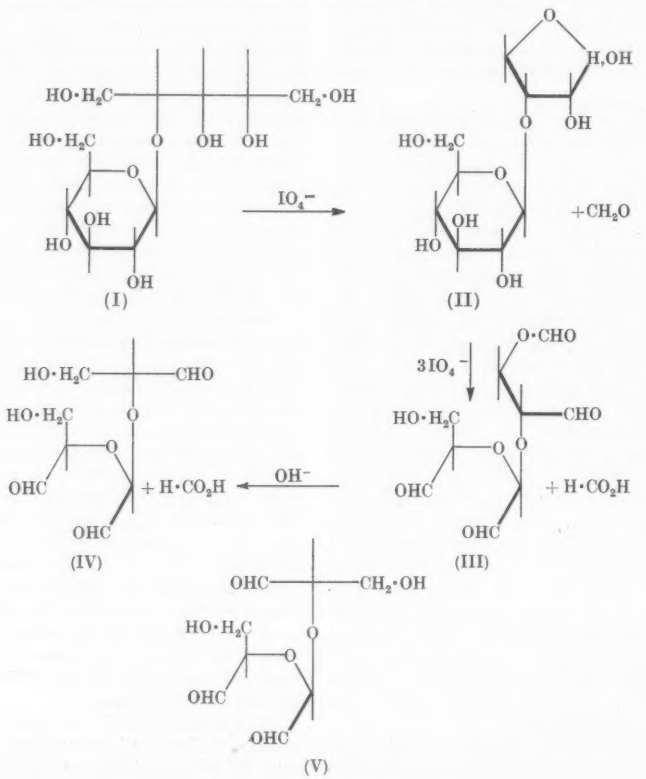
Mild alkali hydrolysis under conditions comparable with those used for the formation of nucleotides from ribonucleic acid had little effect on the phosphodiester linkages in the teichoic acid. However, more vigorous conditions caused complete hydrolysis of phosphodiester linkages, the main products being alanine and a mixture of isomeric phosphomonesters of glucosylribitol. Although these phosphates were not separated from each other on paper chromatograms their structures were determined by methods described later. Phosphate groups were readily removed from this mixture by prostate phosphatase, the resulting crystalline glucosylribitol being isolated in high overall yield.

Acid hydrolysis of the glucosylribitol yielded glucose, ribitol and anhydroribitol; the proportion of anhydroribitol to ribitol increased with

increasing time of hydrolysis. The low negative rotation of the glucosylribitol was consistent with a β-D-glucopyranoside, and this was confirmed by its ready hydrolysis to glucose and ribitol in the presence of a β-glucosidase. This glucoside differs chromatographically (periodate–Schiff reagents) from a glucosylribitol obtained in a similar manner from the ribitol teichoic acid of *L. arabinosus*. The latter is believed to be an α-glucoside, since it has a high positive rotation and is unaffected by a β-glucosidase (unpublished work with Dr A. R. Archibald).

It seemed unlikely that the glucose was attached at the terminal 1 (5)-position of the ribitol since periodate oxidation, followed by reduction of products with sodium borohydride and then acid hydrolysis (Viscontini *et al.* 1955), gave glycerol but no ethylene glycol. Moreover, if the glucosyl residue is attached to position 3 in the ribitol, the compound should give a yellow colour on paper chromatograms sprayed with the periodate–Schiff reagents for glycols (Baddiley, Buchanan & Carss, 1957*a*). The glucosylribitol gave the normal purple colour in this test, thus indicating the absence of the glucose at the 3-position.

The structure 4-*O*-(β-D-glucopyranosyl)-D-ribitol (I) was established for the glucoside by periodate oxidation. It consumed 4 mol.prop. of periodate and gave 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde. It follows that the glucopyranosyl substituent must be at position 2 or 4 on the ribitol residue. The oxidation was carried out with sodium periodate in the absence of buffer, and under these conditions only 1·2 mol.prop. of formic acid was liberated directly. The additional 0·8 mol.prop. of formic acid was released when the solution was made alkaline, and was presumably present as a formic ester in the oxidation mixture. It is concluded that the main course of the oxidation occurs first between carbon atoms 1 and 2 in the ribitol residue to give the 3-*O*-glucoseleythrose (II) and formaldehyde. Further oxidation of this disaccharide would give a formyl ester (III) and 1 mol.prop. of formic acid. A second mol.prop.



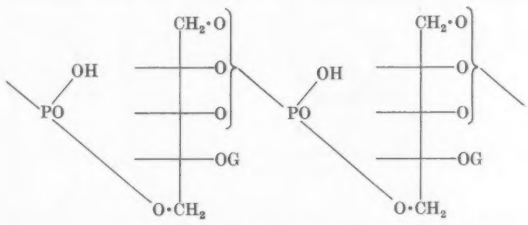
of formic acid would be released under alkaline conditions, the final product being the trialdehyde (IV). The suggested sequence is in agreement with recent views on the course of periodate oxidation of reducing sugars (Warsi & Whelan, 1958; Hough, Taylor, Thomas & Woods, 1958). The preferential oxidation of the acyclic polyol in the first stage of the sequence is supported by Brown, Hall & Letters's (1959) observations on the periodate oxidation of glycerol inositol phosphates. The glycerol residue is readily oxidized under conditions which do not affect the inositol.

Positions 2 and 4 in the ribitol residue can be distinguished from each other by a study of the trialdehyde obtained from the periodate oxidation. 4-*O*-(β-D-Glucopyranosyl)-D-ribitol (I) would give the trialdehyde (IV), whereas 2-*O*-(β-D-glucopyranosyl)-D-ribitol would yield the stereoisomeric compound (V). Both these trialdehydes possess two glyceraldehyde residues; moreover, in both cases the glyceraldehyde residue which arises from carbon atoms 4, 5 and 6 of the original D-glucose will have the D-configuration. The other will have the D-configuration in structure (IV) and the L-configuration in structure (V). It follows that whereas the trialdehyde (IV) would be optically active, that with structure (V) would be a *meso* compound. As all other products of the oxidation are optically inactive, direct measurement of the rotation of the oxidation mixture should be permissible. In fact, this mixture exhibited pronounced optical activity, thus indicating structure (IV) for the trialdehyde. This activity was unaltered after several hours in the presence of acid; these conditions would have effected equilibration of unsymmetrical hemi-acetals of (IV) or (V) which might have arisen during the oxidation.

Further oxidation of the trialdehyde with bromine water, and hydrolysis of the resulting tricarboxylic acid, gave glyceric acid. This acid from structure (IV) would have the D-configuration, whereas from structure (V) it would be racemic. The specific rotation of glyceric acid is considerably enhanced in the presence of molybdate (Isherwood, Chen & Mapson, 1954), and even with the small quantities of material used in this work a marked positive rotation was observed in this way. It follows that the glyceric acid has the D-configuration and must arise from structure (IV); thus the glucosylribitol is correctly represented by structure (I).

The general behaviour of ribitol teichoic acids, and particularly the nature of their products of acid hydrolysis, indicates that they are polymers in which ribitol residues are joined through phosphodiester linkages. These polymers are analogous to the glycerol teichoic acids, in which it is known that glycerol residues are joined to each other through phosphodiester groups. This is now supported by titration studies on the ribitol teichoic acid from *B. subtilis*, after careful removal of alanine residues with cold dilute ammonia. One primary acidic group is present for each phosphorus atom in the polymer. The low but measurable content of secondary acidic groups suggests the presence of a few phosphomonoester residues, but these are believed to represent an end group terminating the polymer chain.

The alkali-lability of this teichoic acid is explained by a structure in which at least one hydroxyl group is adjacent to each phosphodiester group in the polymer. The mechanism of hydrolysis in alkali would be similar to that proposed for ribonucleic acid (Brown & Todd, 1952; Brown, Magrath, Neilson & Todd, 1956), and must involve the intermediate formation of cyclic phosphates. The somewhat more vigorous treatment required for the hydrolysis of teichoic acid reflects the greater difficulty in forming a cyclic phosphate from an open-chain polyol compared with the sterically easier situation in ribonucleic acid. As the alkali hydrolysis of this teichoic acid gives very largely a mixture of two monophosphates of glucosylribitol it is concluded that hydrolytic fission is unidirectional. It follows that each phosphodiester group in the polymer chain bears only one substituent containing an adjacent hydroxyl group. The structure (VI) satisfies this requirement and is strongly supported by biogenetic consideration. It is reasonable to assume that ribitol teichoic acids



(VI) G = β-D-glucopyranosyl

are formed naturally from the bacterial nucleotide cytidine diphosphate ribitol (Baddiley, Buchanan, Carss & Mathias, 1956*a*), the presence of which has been demonstrated in extracts of *B. subtilis* (Clarke, Glover & Mathias, 1959, and unpublished observations in this Laboratory), and that a similar relationship exists between the glycerol teichoic acids and cytidine diphosphate glycerol (Baddiley, Buchanan, Mathias & Sanderson, 1956*c*). Polymer formation through these nucleotides is likely to occur by successive transfer of the polyol phosphate group in the nucleotide. It is known that cytidine diphosphate ribitol is a derivative of D-ribitol 5-phosphate (Baddiley *et al.* 1957*a*), and it follows that one phosphate linkage in ribitol teichoic acids should be at the 5-position in the D-ribitol residues, as shown in (VI).

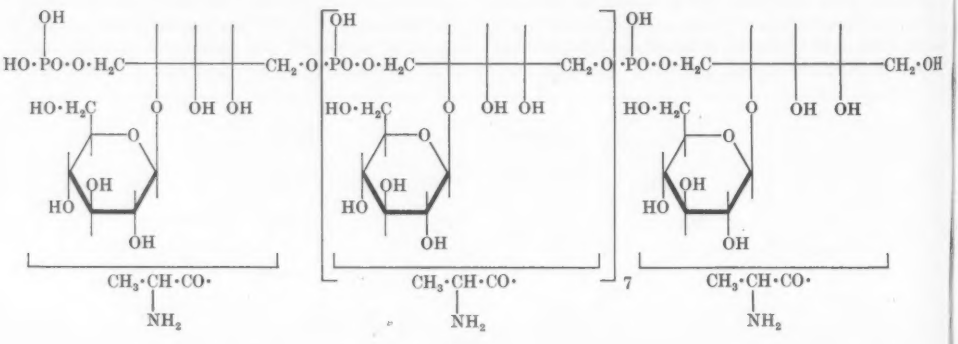
A sample of teichoic acid from which the alanine residues had been removed was oxidized with periodate. Acid hydrolysis of the oxidized product yielded insignificant amounts of anhydroribitol, consequently each ribitol residue in the polymer must contain an unsubstituted α-glycol grouping. From these considerations positions 1 and 5 or 3 and 5 are the only ones available for the phosphodiester linkages in the glucosylribitol residues.

If the phosphodiester linkages are at positions 3 and 5 then each ribitol residue should yield 1 mol.prop. of formaldehyde on oxidation with periodate, whereas a 1:5-phosphodiester would yield formaldehyde only from a glucosylribitol residue at one end of the polymer chain. For every 3 mol.prop. of periodate consumed by the polymer, less than 0·12 mol.prop. of formaldehyde were produced, consequently the teichoic acid must contain only ribitol 1:5-phosphodiester linkages and will have the structure (VII). Although the number of alanine ester residues in this structure is greater than the analysis would indicate, this is intended to represent undegraded teichoic acid.

The course of the alkali hydrolysis of the polymer is readily understood on the basis of structure (VII), which for this purpose can be considered in a simplified form (VIII) still embodying the essential features of the larger molecule. Unidirectional cyclization of the phosphodiester linkages would give the 1:2-cyclic phosphate of glucosylribitol 5-phosphate (IX), the 1:2-cyclic phosphate of glucosylribitol (X) and glucosylribitol (XI). Under the reaction conditions employed the cyclic phosphates would hydrolyse to a mixture of isomeric glucosylribitol diphosphates (XII) and (XIII) and isomeric glucosylribitol phosphates (XIV) and (XV).

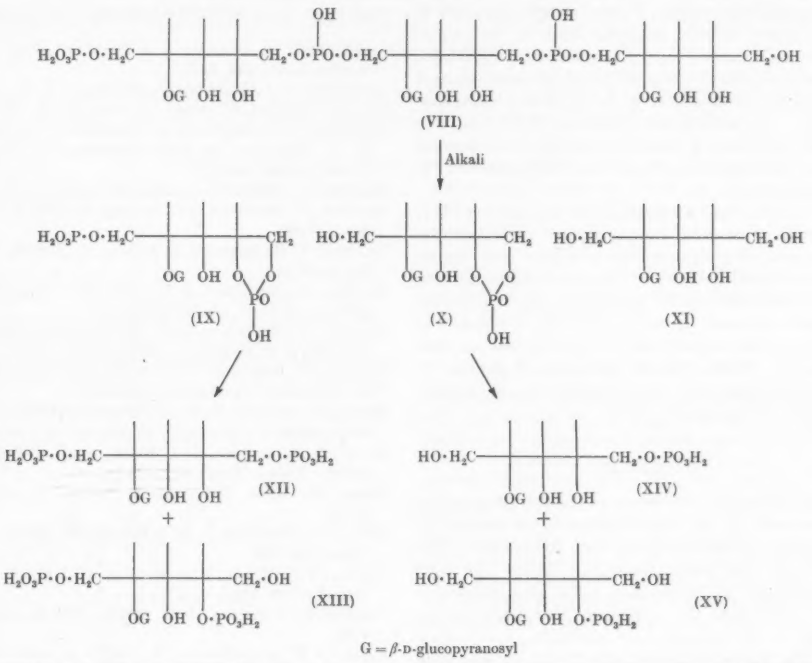
The relative amounts of the five products will depend upon the chain length of the polymer. In fact, the main products were the two monophosphates (XIV) and (XV), arising from the glucosylribitol phosphate residues within the chain. A small amount of glucosylribitol (XI) was observed on paper chromatograms of the hydrolysate, and a phosphate-containing spot was detected in the position expected for the mixture of diphosphates (XII) and (XIII); as these products would arise only from the ends of the chain the amounts formed were small. Although the diphosphates have not yet been fully characterized they give glucosylribitol on treatment with phosphatase. The structure of the mixture of monophosphates has been established by hydrolysis with a β-glucosidase. The products were glucose and a mixture of ribitol 1(5)-phosphate and ribitol 2(4)-phosphate. These ribitol phosphates were conveniently identified by paper chromatography, whereby they can be separated from each other and readily distinguished from the isomeric ribitol 3-phosphate (Baddiley, Buchanan & Carss, 1957*b*).

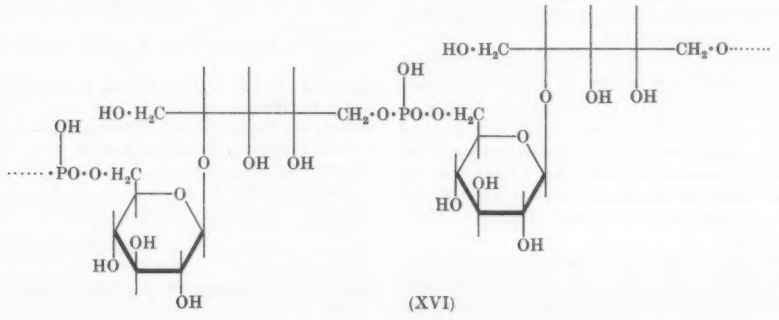
The end groups in structure (VII) are in agreement with the most likely mechanism for the biosynthesis of this teichoic acid. Although the



(VII)

sequence of enzymic reactions between cytidine diphosphate ribitol and the teichoic acid is not yet known, it is highly probable that D-ribitol 5-phosphate residues, with or without alanine and glucose substituents, are transferred to the polymer chain. Thus it is reasonable to expect to find a phosphomonoester group at one end of the molecule. This would account for the observed degradation products and also for the small amount of secondary acidity noted during the titration discussed earlier. As the ratio of primary to secondary acidic groups was 9:1 it follows that the chain comprises eight phosphodiester linkages and one phosphomonester. The presence of a small amount of phosphomonoester groups in the teichoic acid which had had its alanine removed was also demonstrated by the liberation of inorganic phosphate during treatment with prostate phosphomonoesterase. Unfortunately, it was not possible to determine the molecular weight of the polymer in this way, since the liberation of phosphate was very slow and incomplete. The ratio periodate consumed:formaldehyde produced (26:1) and the ratio total phosphorus:formaldehyde produced





(8·8:1) on oxidation of teichoic acid from which alanine has been removed is also consistent with a polymer in which nine glucosylribitol units are joined through phosphodiester linkages, as shown in structure (VII).

At least one of the pneumococci type-specific substances (S. VI) is a ribitol phosphate derivative (Rebers & Heidelberger, 1959*a*). Although the structure of this compound is not yet settled it appears to be a polymer in which a trisaccharide composed of galactose, rhamnose and glucose is attached to the 3-position in the ribitol residues. It is not known whether it is related biochemically to the teichoic acids. Recent work (Rebers & Heidelberger, 1959*b*) suggests that in the type-specific substance the phosphodiester linkages involve a hydroxyl group in the ribitol residues and a hydroxyl at position 2 or 4 of the galactose residues. An analogous structure (XVI) for the teichoic acid from *B. subtilis* is unlikely for several reasons (alanine ester groups have been omitted for convenience).

This is the only alternative to structure (VIII) that would consume 3 mol.prop. of periodate for each repeating polymer unit and give only traces of formaldehyde. It is not easy to understand how such a structure can be formed in any reasonable biogenetic scheme involving cytidine diphosphate ribitol, nor can it yield ribitol diphosphate on acid hydrolysis. Moreover, the absence of glucose 6-phosphate from acid hydrolysates is not readily explained by structure (XVI).

SUMMARY

1. About 60% of the dry weight of *Bacillus subtilis* walls is a ribitol teichoic acid composed of alanine, glucose, ribitol and phosphate. This compound has been separated from the wall mucopeptides by extraction with cold trichloroacetic acid and its structure has been determined.

2. The alanine residues have the D-configuration and are joined to hydroxyl groups in the polymer through their carboxyl groups. These alanine ester linkages are unusually labile.

3. Alkali hydrolysis of the teichoic acid yields a mixture of phosphoric esters of a glucosylribitol. After enzymic removal of phosphate groups a crystalline glucosylribitol was obtained in good yield. The structure 4-*O*-(β-D-glucopyranosyl)-D-ribitol (I) is established for this compound by periodate oxidation, by the action of β-glucosidase and from a study of the optical activity of oxidation products.

4. Periodate oxidation of the teichoic acid from which alanine residues had been removed indicates that the glucosylribitol residues are joined through phosphodiester linkages at positions 1 and 5 in the ribitol.

5. Titration studies and other evidence shows that this teichoic acid has the structure (VII), in which nine alanylglucosylribitol 5-phosphate units are joined together. Biogenetic evidence is consistent with this structure.

We thank Mr A. L. Davison and Miss S. Robinson for assistance in the growth of bacteria, Mr E. H. Boult for assistance in electron microscopy and the Medical Research Council and the Rockefeller Foundation for financial support.

REFERENCES

Armstrong, J. J., Baddiley, J. & Buchanan, J. G. (1959*a*). *Nature, Lond.*, **184**, 248.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. & Greenberg, G. R. (1958). *J. chem. Soc.* p. 4344.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Davison, A. L., Kelemen, M. V. & Neuhaus, F. C. (1959*b*). *Nature, Lond.*, **184**, 247.

Baddiley, J. (1959). *Proc. chem. Soc.* p. 177.

Baddiley, J., Buchanan, J. G. & Carss, B. (1957*a*). *J. chem. Soc.* p. 1869.

Baddiley, J., Buchanan, J. G. & Carss, B. (1957*b*). *J. chem. Soc.* p. 4058.

Baddiley, J., Buchanan, J. G., Carss, B. & Mathias, A. P. (1956*a*). *J. chem. Soc.* p. 4583.

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956*b*). *J. chem. Soc.* p. 2818.

Baddiley, J., Buchanan, J. G., Mathias, A. P. & Sanderson, A. R. (1956*c*). *J. chem. Soc.* p. 4186.

Brown, D. M., Hall, G. E. & Letters, R. (1959). *J. chem. Soc.* p. 3547.

Brown, D. M., Magrath, D. I., Neilson, A. H. & Todd, A. R. (1956). *Nature, Lond.*, **177**, 1124.

Brown, D. M. & Todd, A. R. (1952). *J. chem. Soc.* p. 52.

Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.

Clarke, P. H., Glover, P. & Mathias, A. P. (1959). *J. gen. Microbiol.* **20**, 156.

Consden, R. & Gordon, A. H. (1948). *Nature, Lond.*, **162**, 180.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Foster, A. B., Horton, D. & Stacey, M. (1957). *J. chem. Soc.* p. 81.

Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.

Hough, L., Powell, D. B. & Woods, B. M. (1956). *J. chem. Soc.* p. 4799.

Hough, L., Taylor, T. G., Thomas, G. H. S. & Woods, B. M. (1958). *J. chem. Soc.* p. 1212.

Isherwood, F. A., Chen, Y. T. & Mapson, L. W. (1954). *Biochem. J.* **56**, 54.

Janczura, E., Perkins, H. R. & Rogers, H. J. (1960). *Biochem. J.* **74**, 7P.

Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.

Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181** 149.

Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Rebers, P. A. & Heidelberger, M. (1959*a*). *J. Amer. chem. Soc.* **81**, 2415.
Rebers, P. A. & Heidelberger, M. (1959*b*). *Abstr. Comm. Amer. chem. Soc.* 8C, 17.
Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
Salton, M. R. J. & Ghuysen, J. M. (1957). *Biochim. biophys. Acta*, **24**, 160.
Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
Sharon, N. (1957). *Nature, Lond.*, **179**, 919.
Sharon, N. & Jeanloz, R. W. (1960). *J. biol. Chem.* **235**, 1.
Viscontini, M., Hoch, D. & Karrer, P. (1955). *Helv. chim. acta*, **38**, 642.
Warsi, S. A. & Whelan, W. J. (1958). *Chem. & Ind.* p. 71.

Biochem. J. (1960) **76**, 621

Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

4. A POTENTIOMETRIC METHOD FOR HIGHLY INSOLUBLE COMPLEXES, AND ITS APPLICATION TO COMPLEXES OF ADENINE ANION*

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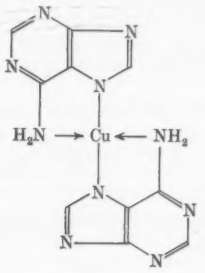
(*Received* 29 *February* 1960)

Adenine, in spite of the 1:2-diamine structure involving the 6- and 7-positions, has little tendency to form complexes with the cations of heavy metals, as was shown quantitatively (Albert, 1953). However, by using less acidic solutions it has been found that the anion of adenine has a strong avidity for bivalent cations, which it binds between $N_{(7)}$ and the primary amino group (Harkins & Freiser, 1958).



(I)

In pursuing this observation, we found that the copper complex was so insoluble that it was necessary to work at a greater dilution than has been customary hitherto in potentiometric work. This necessitated refinements of technique and apparatus which it is believed will be generally useful for insoluble complexes.

This work has revealed that, contrary to what had been supposed, copper binds the adenine anion by almost simultaneous addition of two equivalents, giving the 2:1 complex (I).

* Part 3: Albert (1953).

METHODS

Materials. Glycine (A.R.) was recrystallized from water and dried at 110° for an hour. Adenine (British Drug Houses Ltd.) was recrystallized from water twice and then found to be chromatographically homogeneous. It was recrystallized once more and dried at 110°. Solutions (5 mM) of these substances were prepared in boiled ion-free water. A part of these solutions was diluted in the titration vessel with ion-free water, added under nitrogen from a grade A burette, to yield the concentrations given in the tables.

Pure copper perchlorate and nickel perchlorate solutions were made by pouring a solution of barium perchlorate on to a column of cation-exchange resin (Amberlite IR 120) previously quantitatively converted into the appropriate metal salt. A barium-free fraction of the eluate was collected and standardized by both a gravimetric and an ion-exchange method. Further dilutions were made with boiled ion-free water.

Apparatus. The titration vessel was a 250 ml. tall beaker sealed into a water-jacket through which water from a thermostatically controlled bath was circulated, thus maintaining the solution in the beaker at $20{\cdot}0 \pm 0{\cdot}02°$. The beaker was closed with a rubber bung carrying the electrodes, a nitrogen inlet, a thermometer and a sealable inlet for the glass capillary of a micrometer syringe. The nitrogen inlet was arranged so that a stream of oxygen-free nitrogen (saturated with water) was directed across the surface of the solution.

The pH was measured to 0·002 pH unit with a Vibron electrometer (model 33B; Electronic Instruments Ltd.), complete with pH attachment, glass electrode (type GG 33)

and a saturated calomel electrode (type RJ 23). This instrument, when left under experimental conditions, was found to reproduce a buffer reading to 0·004 pH unit or less, after 24 hr., without readjustment. To prevent precipitation of potassium perchlorate on the sintered-glass barrier, the calomel electrode was placed in an outer jacket fitted with another sintered barrier and containing a solution of 1·60 M-ammonium nitrate and 0·20 M-sodium nitrate adjusted to pH 7 (Perrin, 1958). Ammonium ions passed through this barrier at 59 μg./hr. This was decreased to 3·6 μg./hr. by setting in agar 0·5 ml. of the bridge solution above the sinter. The components were assembled so that the level of the solution in the titration vessel was about 5 mm. below those in the outer jacket and the calomel electrode.

The titration was performed by adding 0·1 N-sodium hydroxide (carbonate free) in small portions from a micrometer syringe. The solution was stirred magnetically during, and for 60 sec. after, each addition. The pH was measured at 60 sec. intervals until constant (usually after about 6 min. and after a drift of about 0·030 to higher pH). When precipitation began, a drift to lower pH occurred; at this point a titration was always discontinued because experience showed that the results obtained were variable and irrelevant.

Calculations. Values of $\bar{n}$ (average number of molecules bound by one atom of metal) and $[L^-]$ (concentration of free chelating species, formerly written [Sc]), were calculated as before (Albert, 1952). These were inserted in equation (1) (Irving & Rossotti, 1953). This is an equation to a straight line and can give results of higher precision than the similar equations used in earlier parts of this series, particularly when $\log K_1 - \log K_2$ lies in the range 0·5–2·5.

$$\frac{\bar{n}}{(\bar{n}-1)[L^-]} = \frac{(2-\bar{n})[L^-]}{(\bar{n}-1)} \cdot \beta_2 - K_1, \qquad (1)$$

where K_1 is the stability constant of the 1:1 complex and β_2 (formerly written K_2) is the overall stability constant of the 2:1 complex (e.g. I). K_2, the constant governing the equilibrium between the 1:1 and the 2:1 complexes, is given by:

$$\log K_2 = \log \beta_2 - \log K_1 \qquad (2)$$

K_1 and β_2 were evaluated from equation (1) by the method of least squares.

When the addition of two equivalents of ligand (to one of the metal) is not stepwise but simultaneous, equation (3) gives consistent results whereas (1) fails to do so (Albert, 1953):

$$\log \beta_2 = \log \bar{n} - \log (2-\bar{n}) - 2 \log [L^-] \qquad (3)$$

The pK_a values of 4·25±0·01 (basic) and 9·83±0·04 (acidic) were found at 20° (5 mM) and used in these calculations.

For the copper concentrations used in this work, the pH regions are such that hydrolysis of the cupric ion is negligible (Dr D. D. Perrin, personal communication; cf. Perrin, 1960).

RESULTS

The refined technique was worked out with glycine, which has a moderately soluble copper salt. It was titrated against alkali at 0·5 mM, with glycine–copper ratios varying from 2:1 to 10:1. The need for the higher ratio arose from the realization that with adenine it would be necessary to work at very low copper concentrations to avoid precipitation. At such low concentrations it was desirable to increase the ligand–metal ratio to force the equilibrium sufficiently far to enable the slope of the formation curve to be studied.

The figures for glycine, given in Table 1, were calculated from equations (1) and (2) as described. To obtain the spread values for the last two columns of Table 1, the mean value of K_1 was substituted in equation (1) and β_2 was solved for ten individual values of $\bar{n}$ and L. Similarly, the mean value of β_2, originally obtained was used to give 12 results for K_1. The first three dilutions in Table 1 gave results which show excellent agreement among one another, thus justifying the use of high ligand–metal ratios. The fourth experiment, where the increased ionic strength corresponds to that obtained in earlier work with 10 mM-glycine (Albert, 1950), shows good agreement, thus justifying titration in dilute solution.

The titration of adenine with nickel (ratio 10:1) by this technique gave $\log K_1$ of 4·37 ± 0·06, as shown in Table 2. Precipitation prevented higher

Table 1. *Determination of the stability constants of the glycine–copper complexes in water at low concentrations of copper*

Concn. of glycine was 0·5 × mM in a total volume of 100 ml. Temp. 20°.

Concn. of Cu^{2+} ions (mM)	Ionic strength (approx.)	$\log K_1$	$\log \beta$	$\log K_2$
0·25	10^{-4}	8·57±0·02	15·70±0·04	7·13±0·03
0·125	10^{-4}	8·60±0·02	15·74±0·05	7·13±0·03
0·05	10^{-4}	8·61±0·02	15·74±0·05	7·13±0·05
0·05	10^{-2} ($NaClO_4$)	8·34±0·03	15·38±0·06	7·05±0·05
		10^{-2} M*-Glycine		
5·0	10^{-2}	8·47	15·38	6·91

* Albert (1950).

Table 2. *Determination of the stability constants of two metal complexes of the adenine anion*

Concn. of adenine, 0·5 mM, and Ni^{2+} ions, 50 μM, in a total volume of 100 ml. Temp., 20°.

Vol. of 0·01 N-NaOH (ml.)	pH	log $[L^-]$	$\bar{n}$	log K_1	
0	6·516				
0·100	7·691	$\bar{6}$·5515	0·122	4·59	
0·150	7·979	$\bar{6}$·8352	0·154	4·43	
0·200	8·146	$\bar{6}$·9981	0·182	4·35	
0·250	8·268	$\bar{5}$·1159	0·210	4·31	
0·300	8·342	$\bar{5}$·1855	0·264	4·37	4·37
0·350	8·414	$\bar{5}$·2531	0·308	4·41	
0·400	8·496	$\bar{5}$·3308	0·326	4·35	
0·500	8·622	$\bar{5}$·4480	0·384	4·35	
0·600	(pptn)	—	—	—	

Adenine 0·10 mM and Cu^{2+} ions 7·5 μM (total volume 100 ml.)

Vol. of 0·01 N-NaOH (ml.)	pH	log $[L^-]$	$\bar{n}$	log K_1 (attempted)	log β	
0	6·259	$\bar{8}$·4182	0·201	6·98	14·11	
0·010	6·359	$\bar{8}$·5164	0·292	7·10	14·20	
0·020	6·447	$\bar{8}$·6018	0·381	7·20	14·17	
0·030	6·519	$\bar{8}$·6698	0·509	7·35	14·19	
0·040	6·579	$\bar{8}$·7260	0·646	7·54	14·22	14·22
0·050	6·633	$\bar{8}$·7762	0·753	7·70	14·23	
0·060	6·684	$\bar{8}$·8227	0·881	—	14·25	
0·070	6·727	$\bar{8}$·8623	0·982	—	14·26	
0·080	(pptn)	—	—	—	—	

values of $\bar{n}$ than 0·38 being attained, even in more dilute solutions, and hence no values of log β and log K_2 could be found.

For the titration of adenine with copper, more dilute solutions were found necessary to avoid precipitation of the copper–adenine complex, which is very poorly soluble. Because of the greater avidity for copper, an $\bar{n}$ value as high as 0·98 was reached without precipitation, as shown in Table 2. When no consistent value for log K_1 could be calculated from equation (1), it was realized that most of the copper cations were simultaneously adding two equivalents of adenine anions. The use of equation (3) confirmed this, and the value log $\beta_2 = 14{\cdot}22 \pm 0{\cdot}05$ was obtained. Even at very low values of $\bar{n}$, little evidence of 1:1 addition could be found.

Qualitative experiments showed that the adenine anion has considerable avidity for all the bivalent cations of the first-transition series of metals.

DISCUSSION

The results obtained with glycine show that potentiometric titration can be adapted for a far greater dilution than has hitherto been used in the determination of the stability constants of metal complexes (see Table 1).

It was shown (Albert, 1953) that the neutral molecule of adenine had even less metal-binding avidity than ammonia. Thus log K_1 for adenine–copper was 2·5, and no detectable combination with cobalt could be found. This earlier work was carried out between pH 3 and 6. But in the present work, performed at higher pH values to furnish significant amounts of adenine anion, a strong avidity for bivalent cations was demonstrated. This avidity lies a little below that shown by the common amino acids. Thus log K_1 for adenine–nickel (4·37) may be compared with log K_1 for glycine–nickel (6·14), and log β_2 for adenine–copper (14·22) may be compared with log β_2 for glycine–copper (15·74).

Our adenine–copper results may be compared with some published figures: log K_1 (7·1), log K_2 (6·4), log β_2 (13·5) (Harkins & Freiser, 1958). These were obtained at concentrations (0·4 mM-adenine and 50 μM-copper) that produced precipitation throughout most of the titration, thus invalidating the results (the solubility product of $[Cu^{2+}]\,[L^-]^2$ is $10^{-19{\cdot}1 \pm 0{\cdot}1}$). There is little stepwise addition of adenine anion to copper, and the two equivalents add simultaneously (for details, see Results section). Simultaneous addition, to which copper is particularly prone, has not previously been encountered in purines but has been demonstrated for riboflavin and several pteridines (Albert, 1953).

SUMMARY

1. The potentiometric technique for the determination of stability constants of metal complexes has been adapted for use in more dilute solutions than has been customary (in one example at 7·5 μM-

metal concentration). The procedure is useful for poorly soluble metal complexes.

2. Application of this technique to the adenine anion confirms that this species, unlike the neutral molecule of adenine, has a high avidity for bivalent cations.

3. Contrary to what has been thought, the 2:1 complex of the adenine anion and copper is formed simultaneously, and not stepwise.

The authors thank Dr D. D. Perrin for his unfailing helpfulness.

REFERENCES

Albert, A. (1950). *Biochem. J.* **47**, 531.
Albert, A. (1952). *Biochem. J.* **50**, 690.
Albert, A. (1953). *Biochem. J.* **54**, 646.
Harkins, T. R. & Freiser, H. (1958). *J. Amer. chem. Soc.* **80**, 1132.
Irving, H. & Rossotti, H. S. (1953). *J. chem. Soc.* p. 3397.
Perrin, D. D. (1958). *J. chem. Soc.* p. 3120.
Perrin, D. D. (1960). *J. chem. Soc.* (in the Press).

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **393rd Meeting** *of the Society was held in the Department of Biochemistry, University College, London, on Friday, 8 April 1960, when the following papers were read:*

COMMUNICATIONS

Possible Precursors for the Synthesis of Muramic Acid by *Staphylococcus aureus* 524. By M. H. Richmond and H. R. Perkins. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Strominger (1958) reported that extracts of *Staphylococcus aureus* and other bacteria could catalyse a reaction between uridine disphospho-*N*-acetylglucosamine and phosphoenolpyruvate, which yielded a product which was thought to be 3-*O*-enolpyruvyl-*N*-acetylglucosamine. No muramic acid was detected. It seemed of interest to see what might be the precursors of the 3-*O*-carboxyethyl side chain of muramic acid during its synthesis by intact organisms. Mandelstam & Rogers (1959) have shown that when *Staphylococcus aureus* is incubated with glucose, alanine, glycine, lysine and glutamic acid it readily forms additional cell-wall material. When the organisms were incubated under these conditions in the presence of generally labelled [^{14}C]glucose (200 μg./ml.) it was found that both the glucosamine and the muramic acid of the isolated cell walls were heavily labelled, whereas labelling in the amino acids was slight.

The specific activity of the two non-carboxyl C-atoms of the side chain of the labelled muramic acid was determined separately. When muramic acid was heated at 100° with 86% sulphuric acid for 17 min. these two carbon atoms yielded acetaldehyde (Strange & Kent, 1959). This acetaldehyde was converted to its 2:4-dinitrophenylhydrazone, which was separated from contaminating material by paper chromatography in light petroleum (b.p. 100–120°)–dimethylformamide (10:3·5, v/v), eluted and estimated by its ultraviolet absorption at 353 mμ. It was found using [^{14}C]-glucose that during the synthesis of cell wall in the absence of added alanine, the specific activity per μg. of carbon in the whole molecule was similar for both glucosamine and muramic acid. When muramic acid was synthesized in the presence of added alanine, the specific activity of its side chain was reduced by 75%. Although lactic acid could be shown to enter the cells, unlike alanine it did not act as a competitor for synthesis of the side chain. In converse experiments it was shown that generally labelled [^{14}C]alanine and [^{14}C]aspartic acid could act as precursors for the muramic acid side chain. When [1-^{14}C]alanine was used the acetaldehyde isolated from muramic acid was not labelled, but it could be shown that radioactivity was present in the carbon monoxide derived from the carboxyl group of muramic acid by the treatment with 86% sulphuric acid. Hence the three carbon atoms of alanine can act as precursors of the muramic acid side chain without inversion. The results are consistent with the idea that pyruvate or phosphoenolpyruvate may be the immediate precursor.

REFERENCES

Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Strange, R. E. & Kent, L. H. (1959). *Biochem. J.* **71**, 333.
Strominger, J. L. (1958). *Biochim. biophys. Acta*, **30**, 645.

Thymidine Kinases in Mammalian Tissues. By S. M. Weissman,* J. Paul, R. Y. Thomson, R. M. S. Smellie and J. N. Davidson. (*Department of Biochemistry, The University, Glasgow*)

Thymidine is converted into thymidine triphosphate (TTP) before incorporation into deoxyribonucleic acid (DNA) in bacterial systems (Bessman, Lehman, Simms & Kornberg, 1958) and in extracts of mammalian cells (Bollum & Potter, 1959; Mantsavinos & Canellakis, 1959; Smellie, Keir & Davidson, 1959).

In the presence of Mg^{2+} and a large excess of adenosine triphosphate, enzymes present in extracts of mammalian cells phosphorylate [^{3}H]thymidine to the 5′-mono-, -di- and -tri-phosphates (TMP, TDP, TTP) which may be separated on ECTEOLA-cellulose (Peterson & Sober, 1956) columns and used as the basis of an enzyme-assay method.

Further purification of the enzymes may be achieved by removal of inactive protein on

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acidification, followed by ammonium sulphate fractionation and chromatography on diethylaminoethylcellulose columns.

The TDP- and TTP-forming enzymes occur in immature rabbit tissues in decreasing order of activity in bone marrow, thymus, appendix, spleen, testes, intestine, buffy coat, kidney, liver, brain, whole blood, plasma and muscle.

In confirmation of the observations of Bollum & Potter (1959) and Mantsavinos & Canellakis (1959), extracts of normal rat liver show little ability to form TDP and TTP. In extracts of rat liver regenerating for 24 hr. after partial hepatectomy, TMP-forming activity is appreciably elevated and TDP-forming activity appears. TDP-forming activity rises to a maximum 30–48 hr. post-operatively, followed by TTP-forming activity.

Disrupted preparations of Earle's 'L' strain of mouse sarcoma cells in tissue culture show a decline in TMP-forming activity shortly after inoculation, followed by a rise to a plateau during growth and a decline on the completion of growth. As the cultures change from lag phase to logarithmic growth there is an increase, first in TDP- and then in TTP-forming activity. The TTP-forming activity shows a moderately sharp peak before completion of growth and this correlates well with measurements of the incorporation of [^{3}H]-thymidine into DNA.

During the early stages of growth the activity of the 'L' cell preparations is not greatly influenced when the cells are grown in medium containing thymidine or 5-fluorodeoxyuridine but after 3 days thymidine kinase activities in cells exposed to thymidine markedly exceed those in control cultures.

REFERENCES

Bessman, M. J., Lehman, I. R., Simms, E. S. & Kornberg, A. (1958). *J. biol. Chem.* **233**, 171.
Bollum, F. J. & Potter, V. R. (1959). *Cancer Res.* **19**, 561.
Mantsavinos, R. & Canellakis, E. S. (1959). *J. biol. Chem.* **234**, 628.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Smellie, R. M. S., Keir, H. M. & Davidson, J. N. (1959). *Biochim. biophys. Acta*, **35**, 389.

Studies on the Biosynthesis of Ribonucleic Acid by Particle-free Extracts of Mammalian Cells.

By R. H. Burdon and R. M. S. Smellie. (*Department of Biochemistry, The University, Glasgow*)

Completely disrupted preparations of Ehrlich ascites carcinoma cells have been found capable of incorporating [5:6-^{3}H]uridine into ribonucleic acid (RNA), and the particle-free extracts obtained by centrifuging these preparations at 105 000 *g* have also been found to incorporate uridine into RNA. The optimum pH for this reaction is 7·9. Adenosine triphosphate, Mg^{2+}, K^+, diphosphopyridine nucleotide (DPN) and glucose are required, and the process involves the formation of radioactive uridine 5′-mono-, -di- and -tri-phosphates (UMP-5′, UDP-5′ and UTP-5′).

Current theories on the biosynthesis of RNA suggest two possible mechanisms whereby uridine could be incorporated: (*a*) phosphorylation to UTP-5′ and addition to an existing polynucleotide with the elimination of inorganic pyrophosphate; (*b*) phosphorylation to UDP-5′ and addition to an existing polynucleotide with the elimination of inorganic orthophosphate. In the system under investigation, addition of either ortho- or pyrophosphate diminishes incorporation of uridine into RNA. This suggests the presence of both mechanisms. Alkaline degradation of the labelled RNA, with subsequent paper chromatography of the products, reveals the presence of 55% of radioactivity in UMP-3′ (-2′) and 20% in uridine, suggesting the occurrence of both terminal and non-terminal incorporation of uridine. In the presence of pyrophosphate, incorporation into the terminal uridine moiety of RNA is depressed, whilst orthophosphate reduces non-terminal incorporation into UMP. During the period of incorporation 20% of the uridine is converted into cytidine derivatives.

Inclusion in the incubation mixtures of either non-radioactive UDP-5′ or UTP-5′, as possible precursors, instead of diluting the resultant radioactivity of RNA, augment it. Together they have little effect. This could be due to a possible coenzymic role of uridine derivatives in the system. However, an equimolar mixture of triphosphopyridine nucleotide (TPN) and DPNH can replace glucose, DPN and UTP-5′ in incubation mixtures. Under these conditions, addition of either non-radioactive UDP-5′, or UTP-5′, decreases the incorporation of uridine into RNA, providing further evidence for the possible existence of two biosynthetic systems in these extracts.

Evidence of net synthesis of RNA has been obtained in the presence of the triphosphates of adenosine, cytidine, guanosine and uridine, Mg^{2+}, TPN and DPNH. An increase in the region of 18%, or 15 μg. per reaction vessel, was observed. When in this experiment the ribonucleoside triphosphates were replaced by the corresponding diphosphates, the net synthesis was slightly lower.

Metabolism of Oxalate in Oxalate-grown *Pseudomonas oxalaticus* (OX 1). By J. R. QUAYLE, D. B. KEECH and G. A. TAYLOR. (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

Previous experiments with oxalate-grown *Pseudomonas oxalaticus* (Quayle & Keech, 1959, 1960) have indicated that the primary step in the synthesis of cell constituents from oxalate is its reduction to glyoxylate. Further work with cell-free extracts of the organism has now demonstrated the formation of oxalyl-coenzyme A and its reduction by reduced triphosphopyridine nucleotide (TPNH) to glyoxylate.

Oxalyl-coenzyme A has been implicated in the decarboxylation of oxalate in an oxalate-grown bacterium by Jakoby, Ohmura & Hayaishi (1956) who suggested the following series of reactions:

(i) Acetyl-CoA + oxalate $\rightleftharpoons$ oxalyl-CoA + acetate;

(ii) Oxalyl-CoA $\xrightarrow{\text{TPP}}$ formyl-CoA + CO_2;

(iii) Formyl-CoA + H_2O $\rightarrow$ formate + CoA.

We find a rapid decarboxylation of oxalate to formate and carbon dioxide in crude extracts of oxalate-grown *P. oxalaticus* on anaerobic incubation with oxalate and adenosine triphosphate. The co-factor requirements for this reaction, using the protein fraction precipitated from extracts by ammonium sulphate at 80% saturation, followed by dialysis, are: adenosine triphosphate, coenzyme A, succinate, Mg^{2+}, and thiamine pyrophosphate (TPP). Substitution of acetate for succinate greatly reduces the reaction rate. Catalytic quantities of succinyl-coenzyme A replace adenosine triphosphate, succinate and coenzyme A. Both oxalohydroxamic and formylhydroxamic acids are formed in the incubation mixture. These results indicate that the decarboxylation of oxalate follows reactions (i)–(iii) except that acetyl-coenzyme A is replaced in (i) by succinyl-coenzyme A, and in addition, a transferase is present capable of transferring the coenzyme A grouping from formyl-coenzyme A to either succinate or oxalate.

An enzyme, purified 20-fold by fractionation with ammonium sulphate, has been obtained from the cell-free extracts and catalyses a TPN- and coenzyme A-dependent oxidation of glyoxylate to oxalate. This indicates, by analogy with the oxidation of acetaldehyde to acetyl-coenzyme A in *Clostridium kluyveri* (Burton & Stadtman, 1953), that the following reactions take place:

(iv) glyoxylate + TPN + CoA $\rightleftharpoons$ oxalyl-CoA + TPNH;

(v) oxalyl-CoA + H_2O $\rightarrow$ oxalate + CoA.

Since the reaction requires only catalytic quantities of coenzyme A and the only reaction product detected is oxalate, it is assumed that oxalyl-coenzyme A hydrolyses either enzymically or spontaneously at pH 8·5, the optimum for the oxidation. Indirect evidence indicates the ready reversibility of (iv), which could thus constitute the key reaction for the primary reduction of oxalate by this organism.

REFERENCES

Burton, R. M. & Stadtman, E. R. (1953). *J. biol. Chem.* **202**, 873.

Jakoby, W. B., Ohmura, E. & Hayaishi, O. (1956). *J. biol. Chem.* **222**, 435.

Quayle, J. R. & Keech, D. B. (1959). *Nature, Lond.*, **183**, 1794.

Quayle, J. R. & Keech, D. B. (1960). *Biochem. J.* (in the Press).

Methanol and Carbon Dioxide Incorporation by *Pseudomonas* sp. AM1. By J. R. QUAYLE and D. PEEL. (*M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*)

An organism, *Pseudomonas* sp. AM1, has been isolated which can grow aerobically on methanol or formate as sole source of carbon and energy. It is a pink, Gram-negative, motile rod with a single polar flagellum, and is similar to both *Pseudomonas methanica* (Dworkin & Foster, 1956) and *Pseudomonas* sp. PRL-W4 (Kaneda & Roxburgh, 1959*a*) in morphology, colour and oxidative properties, but differs from both of them in the range of compounds which support growth.

The path of carbon assimilation in methanol-grown *Pseudomonas* sp. AM1 has been studied by following incorporation of [^{14}C]methanol and [^{14}C]bicarbonate into the ethanol-soluble fractions of bacterial suspensions. The distribution of radioactivity within the non-volatile constituents of these fractions has been analysed by chromatography and radioautography. When either [^{14}C]-methanol or [^{14}C]bicarbonate was used the percentage of the total incorporated radioactivity that was present in amino acids initially decreased, while that in malate, phosphorylated compounds, citrate and polysaccharide initially increased with time. The radioactivity incorporated into the

amino acid fraction in 15 sec. from [^{14}C]methanol was all in serine, as found by Kaneda & Roxburgh (1959*b*, *c*), whereas that from [^{14}C]bicarbonate appeared in glycine and serine in a ratio of 6:4, respectively.

There was no stimulation by ribulose 1:5-diphosphate of the incorporation of [^{14}C]bicarbonate by cell-free extracts of the organism. This fact, together with the kinetic analysis of isotope incorporation into whole cells, shows that a Calvin cycle of carbon dioxide fixation (Bassham *et al.* 1954) is not in operation.

The specific radioactivity of cellular material obtained from cells grown on [^{14}C]methanol in air was reduced by half on bubbling air–carbon dioxide (95:5, v/v) through the growing culture. This shows that at least 50% of the methanol carbon is assimilated as carbon dioxide.

These results suggest that growth of *Pseudomonas* sp. AM1 on methanol involves carbon dioxide fixation into a C_2 compound and condensation of the C_2 compound with a C_1 unit, derived from methanol, to give a C_3 compound. It is not yet known whether glycine and serine are these C_2 and C_3 compounds, or whether they are reflections of undetected primary intermediates such as glyoxylate and hydroxypyruvate.

REFERENCES

Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. E., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.

Dworkin, M. & Foster, J. W. (1956). *J. Bact.* **72**, 646.

Kaneda, T. & Roxburgh, J. M. (1959*a*). *Canad. J. Microbiol.* **5**, 87.

Kaneda, T. & Roxburgh, J. M. (1959*b*). *Canad. J. Microbiol.* **5**, 187.

Kaneda, T. & Roxburgh, J. M. (1959*c*). *Biochim. biophys. Acta*, **33**, 106.

A Comparison of Some Malic Dehydrogenases. By C. J. R. Thorne (introduced by M. Dixon).
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Malic dehydrogenases (MDH) have been isolated from five sources, horse heart, pig heart, rat-liver mitochondria, the supernatant fraction obtained by centrifuging a rat liver homogenate at 10 000 *g* for 30 min., and baker's yeast. In each case a purification of 100- to 200-fold was effected by procedures including ammonium sulphate and alcohol fractionations, calcium phosphate gel adsorption, and chromatography on Amberlite IRC-50 resin and diethylaminoethylcellulose. The preparations were compared in a number of ways.

The use of Amberlite resin, eluting with a concentration gradient of sodium phosphate buffer, pH 7·0, 0·025–0·20M, showed that the rat-supernatant MDH was unretarded, whereas the other four enzymes appeared in the eluate when the buffer concentration reached 0·08–0·11M. Chromatography of the enzymes on diethylaminoethylcellulose, eluting with a concentration gradient of 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 8·2, 0·025–0·25M, permitted a separation into three classes. MDH from horse, pig or rat-mitochondria was unretarded; rat-supernatant MDH appeared in the 0·10–0·13M eluates, and the yeast MDH appeared in the 0·14–0·18M eluates.

The criteria used for comparison of enzymic properties included the Michaelis constants for, and relative rates of oxidation of, L-malate, L-tartrate and *meso*tartrate, the relative rates of reduction of diphosphopyridine nucleotide and four of its analogues (a generous gift from Professor N. O. Kaplan), and the kinetics of oxaloacetate reduction over a pH range. Again horse, pig and rat-mitochondrial enzymes were closely similar, but differed from rat-supernatant MDH and from yeast MDH. The demonstration of the individuality of the two rat-liver enzymes confirmed recent work of Delbrück, Schimassek, Bartsch & Bücher (1959), although the oxaloacetate reduction kinetics differed from those reported.

Homogeneous preparations of MDH from horse and pig were obtained in quantities sufficient for further comparisons to be made. Investigation of their total amino acid composition, *N*-terminal amino acid, sedimentation constants, and absorption spectra showed them to be identical, within the limits of error.

Thus by a variety of criteria, a comparison of the malic dehydrogenases from five sources showed that the enzymes of horse heart, pig heart and rat-liver mitochondria were very similar, but differed from that of rat-liver 'supernatant', and from the yeast enzyme.

This investigation was carried out during the tenure of a D.S.I.R. Research Studentship.

REFERENCE

Delbrück, A., Schimassek, B., Bartsch, K. & Bücher, T. (1959). *Biochem. Z.* **331**, 297.

Purification and Some Kinetic Properties of Ox-brain Lactic and Malic Dehydrogenase. By ALFRED D. WINER* (introduced by H. MCILWAIN). (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

Since the histochemical investigation by Strominger & Lowry (1955) of lactic (LDH) and malic (MDH) dehydrogenase in rabbit brain, no attempt has been made to purify and characterize these pyridine nucleotide-dependent enzymes from cerebral tissues. The present investigation describes the partial purification of these enzymes from ox brain and reports some kinetic and fluorescent (Winer, 1960) properties with reduced diphosphopyridine nucleotide (DPNH) and reduced substrate and substrate analogues.

Purification involved the following steps. (1) Homogenization with Na_2HPO_4–NaH_2PO_4–KCl mixture (pH 7·1, *I* 2) and centrifugation at 20 000 ***g***; (2) adsorption on and elution from calcium phosphate gel; (3) $(NH_4)_2SO_4$ precipitation; (4) heat treatment in the presence of DPNH and reduced substrate analogue; and (5) chromatography on diethylaminoethylcellulose. Based on turnover numbers, the LDH is purified over 100-fold and the MDH about 80-fold. Both enzymes are stable only when dialysed for prolonged periods against ethylenediaminetetra-acetic acid and cysteine. The extinction ratio 280 mμ:260 mμ of the purest LDH fraction is 1·6 and the turnover number 8500 moles of DPN/135 000 g. protein/min. at pH 9·6 in 0·1 M-glycine–NaOH at 25°. The molecular weight is assumed to be that of the beef-heart enzyme (Neilands, 1954). The enzyme is specific for L-lactate and shows no activity toward D-lactate or D- or L-malate. The pH optimum from the lactate–DPN side is 9·0 in 0·1 M-glycine–NaOH and shows no pH optimum from the pyruvate–DPNH side above pH 6 if pyruvate is held below inhibiting concentrations. The K_m for pyruvate at pH 7 (DPNH = 3 μM) is 30 μM and for L-lactate at pH 9·6 (DPN = 100 μM) is 5 mM. The enzyme is inhibited by oxalate and the inhibition is competitive with respect to lactate; oxamate inhibits competitively with pyruvate. *p*-Chloromercuribenzoate in a final concentration of 0·1 mM inhibits the enzyme by 50 % at pH 7, but pre-incubation with coenzyme protects against loss of activity.

The purest MDH fraction has an extinction ratio 280 mμ:260 mμ of 1·4 and a turnover number of 3500 moles of DPN/40 000 g. of protein/min. at pH 9·6. The molecular weight is assumed to be that of the pig-heart mitochondrial enzyme (Wolfe & Neilands, 1956). The enzyme is specific for L-malate and shows no activity with the D-isomer. The pH optimum from the L-malate–DPN side is pH 10 in 0·1 M-glycine–NaOH. The K_m for L-malate at pH 9·6 (DPN = 100 μM) is 100 μM, and for oxaloacetate at pH 7 (DPNH = 2 μM) 20 μM. The enzyme is inhibited by high concentrations of D-malate and this inhibition is competitive with respect to L-malate. *p*-Chloromercuribenzoate in a final concentration of 0·05 mM inhibits the enzyme by 50 % at pH 7.

The author wishes to thank Professor H. McIlwain, in whose laboratory this investigation was performed, for his encouragement and helpful advice.

REFERENCES

Neilands, J. B. (1954). *J. biol. Chem.* **208**, 225.
Strominger, J. L. & Lowry, O. N. (1955). *J. biol. Chem.* **213**, 635.
Winer, A. D. (1960). *Biochem. J.* **76**, 5P.
Wolfe, R. G. & Neilands, J. B. (1956). *J. biol. Chem.* **221**, 61.

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Fluorescent Studies of Ox-brain Lactic and Malic Dehydrogenase. By ALFRED D. WINER (introduced by H. MCILWAIN). (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

Similar to the previous reports for heart-muscle and rat-liver lactic dehydrogenase (LDH) (Winer, Novoa & Schwert, 1957; Winer, Schwert & Millar, 1959), the ox-brain enzyme shows a spectral shift and augmentation in the fluorescent spectrum to 440 mμ when reduced diphosphopyridine nucleotide (DPNH) (fluorescent maximum at 460 mμ when activated at 340 mμ) combines with the protein. When L-lactate, in saturating amounts, is added to the LDH–DPNH complex at pH 7, there is a further increase in fluorescence and shift in wavelength to 430 mμ. A highly fluorescent pH-dependent ternary complex of LDH, DPNH and oxalate is observed when oxalic acid is added to the LDH–DPNH complex, with a further wavelength shift to 420 mμ and an apparent dissociation constant (K_I') of 5×10^{-4} M at pH 7 in phosphate (*I* 0·1), 25°. Potassium oxamate quenches the fluorescence of LDH–DPNH and at pH 7 the K_I' is 10^{-4} M, and at pH 10 in 2-amino-2-hydroxymethyl-

propane-1:3-diol–potassium chloride (I 0·2) the K'_I is 10^{-2}M. The enzyme concentration is obtained by titration with an excess of DPNH in the presence of an excess of oxalate. From titration data at pH 7, approximately 3 moles of DPNH react per mole of LDH, assuming an absorbancy index at 280 mμ of $19{\cdot}3 \times 10^4$ cm./mole/l. (Takenaka & Schwert, 1956). The formation of binary and ternary complexes can also be seen from the enhancement of activation spectra at 280 mμ when the fluorescent wavelength is held constant at 430 mμ. Hydroxymalonate and dihydroxyfumarate, inhibitors of brain respiration *in vitro* (Jowett & Quastel, 1937), which are not substrates for LDH, form ternary complexes with LDH–DPNH and exhibit a wavelength shift to 425 mμ.

The malic dehydrogenase (MDH) fraction shows a wavelength shift and augmentation in the fluorescence spectrum to 450 mμ when it combines with DPNH at pH 6. The addition of L-malate to MDH–DPNH shows a transient ternary complex with a shift in wavelength to 445 mμ. D-Malate, which has been shown to form a highly fluorescent ternary complex with pig-heart MDH–DPNH and a wavelength shift to 395 mμ (Theorell & Langan, unpublished results), shows a similar pH-dependent ternary complex with the ox-brain enzyme. The K'_I at pH 7 in phosphate (I 0·1) and 25° is 10^{-4}M and increases markedly as the pH increases. The enzyme concentration is determined by titration with an excess of DPNH at pH 6 in the presence of an excess of D-malate. At this pH, approximately 1 mole of DPNH combines per mole of MDH assuming an absorbancy index at 280 mμ of $1{\cdot}84 \times 10^4$ cm./mole/l. (Wolfe & Neilands, 1956). No ternary complex formation is observed with either oxalate or oxamate, nor does fumarate, malonate or citrate at 10^{-3}M have any observable effect on the fluorescence of MDH–DPNH at pH 6.

REFERENCES

Jowett, M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 275.

Takenaka, Y. & Schwert, G. B. (1956). *J. biol. Chem.* **223**, 157.

Winer, A. D., Novoa, W. B. & Schwert, G. W. (1957). *J. Amer. chem. Soc.* **79**, 657.

Winer, A. D., Schwert, G. W. & Millar, D. B. S. (1959). *J. biol. Chem.* **234**, 1149.

Wolfe, R. G. & Neilands, J. B. (1956). *J. biol. Chem.* **221**, 61.

The Effect of Human-Serum Albumin on the Oxidation of Glutathione in Human Erythrocytes.

By P. C. Jocelyn. (*Department of Clinical Chemistry, University of Edinburgh, Royal Infirmary, Edinburgh*)

When human erythrocytes depleted of glucose are oxygenated in a solution of human-serum albumin (fraction V) (HSA), the rate of intracellular oxidation of glutathione (GSH) is greater than for the same cells oxygenated in normal saline. This stimulant effect of HSA reaches a maximum value at 0·6–0·8 g./100 ml. of solution and is not observed with a solution of bovine-serum albumin (fraction V). The oxyhaemoglobin content of the oxygenated cells (determined with the Brinkmann Haemoreflector) decreases with increasing concentration of HSA (up to at least 4%, w/v) in the medium, so that the oxygen tension within the cell probably bears no direct relationship to the rate of oxidation of GSH.

The effect of treating cells with carbon monoxide on this rate of oxidation has been investigated. There is a substantial increase (20–50%) in the rate both in saline and also in HSA over the corresponding rate in the untreated cells. This increase is by about the same amount as the gas elevates the original cell level of GSH (Numata, 1940) over that in the untreated cells. Using different batches of untreated cells, there is found to be a significant correlation between the initial level of GSH and its subsequent rate of oxidation in saline. On this evidence it is suggested that the effect of carbon monoxide in increasing the rate of oxidation of GSH may be due to its releasing GSH from some complexed form in the intact cell.

The stimulant effect of HSA is not affected by previous oxidation of the protein thiol group.

Most of the GSH oxidized in these experiments is converted into the oxidized form (GSSG), but there is also a part unaccounted for. This loss is channelled through GSSG because incubation of cells containing preformed GSSG results in its progressive loss, probably by a non-oxidative route.

REFERENCE

Numata, I. (1940). *Biochem. Z.* **304**, 404.

The Thiol Group in the 'Active Site' of Creatine Phosphokinase. By D. C. Watts, B. R. Rabin and E. M. Crook. (*Department of Biochemistry, University College, London, W.C.* 1)

Creatine phosphokinase has been shown to be a thiol enzyme (Ennor & Rosenberg, 1954; Benesch, Lardy & Benesch, 1955). In order to help to elucidate the role of the thiol group in the catalysis we have investigated the kinetics of the reaction of the crystalline enzyme (Kuby, Noda & Lardy, 1954) with iodoacetate. The reaction was followed by measuring both iodide production and loss of kinase activity. The reaction rate depends markedly on ionic strength, but varies little with pH over the range 6–10. At pH 9·0 and ionic strength 0·12 the rate of inhibition with iodoacetate was reduced in the presence of a mixture of adenosine triphosphate (ATP) and Mg^{2+}, but was unaffected by ATP, Mg^{2+} or creatine alone or a mixture of creatine and Mg^{2+}. However, if all three substrates (Mg^{2+}, ATP and creatine) were simultaneously present and the enzymic reaction allowed to proceed to equilibrium, then marked protection from iodoacetate inhibition was observed by this equilibrium mixture. Thus the equilibrium mixture decreases the reactivity of the thiol group at the active site towards iodoacetate. These facts are consistent with an interaction of the thiol group in the transition state with the Mg^{2+}–ATP component of the reaction system. Alternative mechanisms are also possible and further work is in progress to elucidate this problem.

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REFERENCES

Benesch, R. E., Lardy, H. A. & Benesch, R. (1955). *J. biol. Chem.* **216**, 663.
Ennor, A. G. & Rosenberg, N. (1954). *Biochem. J.* **57**, 203.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.

The Demonstration of a 16-Hydroxy Steroid Dehydrogenase in Rat Kidney. By R. J. B. King. (*Department of Biochemistry, University of Edinburgh, Edinburgh* 8)

During investigations on the ability of various rat tissues to metabolize oestriol it was discovered that kidney homogenates supplemented with di- or tri-phosphopyridine nucleotide could metabolize appreciable amounts of oestriol without producing any 2-hydroxyoestriol (King, 1960).

Two phenolic metabolites were detected, metabolite I having the same mobility as 16-oxo-oestradiol-17β and the other the same as 16-*epi*oestriol in the formamide–chloroform system. Metabolite I, formed in about 20% yield was ketonic, whilst II (1% yield) was not ketonic. These metabolites were separated by reaction with Girard's reagent and purified by chromatography in formamide–chloroform. Metabolite II was further purified by chromatography on a Celite column using 70% aqueous methanol–ethylene dichloride (Marrian & Bauld, 1955), the fraction corresponding to 16-*epi*oestriol being collected. This fraction contained all of metabolite II and its behaviour in the Kober reaction was the same as 16-*epi*oestriol. It had the same mobility as 16-*epi*oestriol when chromatographed on paper in 70% aqueous acetic acid–ethylene dichloride.

The infrared spectrum of I was almost identical with that of 16-oxo-oestradiol-17β. The Fast Black Salt K of I (Heftmann, 1950) had the same mobility as the Fast Black Salt K of 16-oxo-oestradiol-17β in the system ethanol–water–toluene–light petroleum (3:7:20:10, by vol.).

Under similar experimental conditions 16-*epi*-oestriol gave 5% of 16-oxo-oestradiol-17β and 1% of oestriol, while 17-*epi*oestriol gave 15% of a ketonic and 1% of a non-ketonic product with the same mobilities in formamide–chloroform as 16-oxo-oestradiol-17β and oestriol, respectively. However, the non-ketonic compound was less polar than oestriol when chromatographed in benzene–methanol–water (20:11:9, by vol.) for 24 hr. This chromatographic behaviour is the same as that of 16:17-*epi*oestriol (Breuer, Knuppen & Pangels, 1959). It would seem probable that the metabolites formed from 17-*epi*oestriol are 16-oxo-oestradiol-17α and 16:17-*epi*oestriol.

In no case could any 16α-hydroxyoestrone or 16β-hydroxyoestrone be detected, even though the chromatographic system used gave a good separation of these two compounds and 16-oxo-oestradiol-17β. This indicated that the 17-hydroxyl group of the three steroids tested was not attacked under these conditions. Rat-liver, ovary and uterus homogenates did not show this 16-hydroxy dehydrogenase activity, but rat-liver microsome plus supernatant fraction can produce small amounts of 16-oxo-oestradiol-17β from oestriol. This kidney 16-hydroxy dehydrogenase activity is localized in the supernatant fraction, and either DPN or TPN is required for this activity.

REFERENCES

Breuer, H., Knuppen, R. & Pangels, G. (1959). *Hoppe-Seyl. Z.* **317**, 248.
Heftmann, E. (1950). *Science*, **111**, 571.
King, R. J. B. (1960). *Biochem. J.* **74**, 22P.
Marrian, G. F. & Bauld, W. S. (1955). *Biochem. J.* **59**, 136.

Effects of Penicillin on a Mutant of *Escherichia coli* requiring Diaminopimelic Acid. By PAULINE MEADOW. (*Department of Biochemistry, University College, London, W.C. 1*)

When a mutant of *Escherichia coli* requiring both diaminopimelic acid and lysine for growth has exhausted all the diaminopimelic acid from the growth medium, the cells lyse. In hypertonic media, exhaustion of diaminopimelic acid, or the addition of penicillin, causes formation of spheroplasts from exponentially growing cells (Meadow, Hoare & Work, 1957). The effects of deprivation of diaminopimelic acid and of penicillin have now been compared more closely.

Cells growing exponentially in a medium containing inorganic salts, glucose, diaminopimelic acid and lysine were harvested at half-maximal growth and distributed into flasks containing the inorganic salt medium with and without amino acid supplements. Glucose was added to initiate growth and the cultures were shaken vigorously for 1 hr. at 37°, with or without the addition of penicillin. Portions of the suspensions were removed at 10 min. intervals and viable counts were determined. The optical densities of the suspensions were determined at 680 mμ and, after removing the cells by centrifugation, the optical density of the supernatant fluid was measured at 260 mμ. Rapid decrease in optical density at 680 mμ occurred in all flasks containing penicillin, while without penicillin, deprivation of diaminopimelic acid caused slower lysis. As had been shown by Trucco & Pardee (1958) with *E. coli* B, penicillin caused immediate release of material absorbing at 260 mμ. There was also an immediate fall in viable counts in all flasks containing penicillin. In the control flasks, without penicillin, viable counts fell only after exhaustion of diaminopimelic acid from the medium.

Cystine, a competitive inhibitor of diaminopimelic acid, partially protected the mutant from attack by penicillin in the complete growth medium (containing diaminopimelic acid and lysine), but had no effect when the growth was not maximal. Lanthionine, which protects against lysis caused by deprivation of diaminopimelic acid, did not alter the lytic action of penicillin.

The incorporation of [^{14}C]glucose, [^{14}C]lysine and [^{14}C]diaminopimelic acid into fractionated cell walls and intracellular proteins was investigated with and without penicillin. In the first 30 min. after addition of penicillin to cells growing exponentially, viable counts fell and material absorbing at 260 mμ was released, but there was no change in the amounts of glucose, lysine or diaminopimelic acid incorporated into cell walls or intracellular protein.

REFERENCES

Meadow, P., Hoare, D. S. & Work, E. (1957). *Biochem. J.* **66**, 270.

Trucco, R. E. & Pardee, A. B. (1958). *J. biol. Chem.* **230**, 435.

Selective Inhibition by 2-Heptyl-4-hydroxyquinoline *N*-oxide of some Peroxidative Reactions Catalysed by Certain Haemoproteins and by a Flavoprotein. By M. KOGUT and J. W. LIGHTBOWN. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The streptomycin antagonist, 2-heptyl-4-hydroxyquinoline *N*-oxide, a potent inhibitor of certain cytochrome-electron-transport systems, was shown to inhibit the succinoxidase and reduced diphosphopyridine nucleotide (DPNH) oxidase activities of heart-muscle sarcosomes. Oxidation of cytochrome *b* and reduction of cytochrome *c* were inhibited. Similar effects were observed with *Bacillus subtilis* (Lightbown & Jackson 1956; Jackson & Lightbown, 1958).

Concentrations of the heptylquinoline *N*-oxide producing the above effects have now been found to inhibit strongly the DPNH peroxidase of *B. subtilis*, recently isolated and purified (Lightbown & Kogut, 1959). This enzyme is a flavoprotein, containing no haem groups. The inhibition was non-competitive with either DPNH or hydrogen peroxide and had K_i 1·6μM.

In an earlier attempt to study the mode of action of this inhibitor by means of a model system, it was found that a peroxidative oxidation of reduced 2:6-dichlorophenolindophenol by hydrogen peroxide or methyl hydrogen peroxide in the presence of catalytic concentrations of horse-heart cytochrome *c* or of haematin, was also inhibited by this material, though at higher concentrations than the above reactions. Antimycin A and substituted naphthaquinones did not have this effect.

Another haemoprotein, the cytochrome 553, isolated from *Pseudomonas* strain KB1 (Kogut, 1957) could also catalyse the oxidation of reduced 2:6-dichlorophenolindophenol by methyl hydrogen peroxide, but this reaction was very little affected by the heptylquinoline *N*-oxide. Nor was the peroxidation of reduced 2:6-dichlorophenolindophenol by horse-radish peroxidase and hydrogen peroxide inhibited, using a range of concentrations of dye, peroxide and inhibitor.

It was further found that whilst the reduction of cytochrome *c* by reduced 2:6-dichlorophenolindophenol or ascorbic acid was not affected by the inhibitor, the oxidation of reduced cytochrome *c* by hydrogen peroxide, and under certain conditions by heart-muscle preparations, could be inhibited by this material.

An attempt to obtain direct evidence of an interaction between cytochrome *c* and the heptylquinoline *N*-oxide indicated that any such interaction was spectroscopically indistinguishable from oxidation of cytochrome *c*.

Some implications of these observations for the mode of action of the substituted quinoline *N*-oxides have been considered. In particular, it should be noted that there is no justification for using inhibitory effects of this material on oxidative systems as diagnostic evidence for the participation of cytochromes.

REFERENCES

Jackson, F. L. & Lightbown, J. W. (1958). *Biochem. J.* **69**, 63.
Kogut, M. (1957). *Biochem. J.* **65**, 35P.
Lightbown, J. W. & Jackson, F. L. (1956). *Biochem. J.* **63**, 130.
Lightbown, J. W. & Kogut, M. (1959). *Biochem. J.* **73**, 14P.

Bacterial Oxidation of Protocatechuic Acid. By S. Trippett, S. Dagley and D. A. Stopher. (*Departments of Organic Chemistry and Biochemistry, University of Leeds*)

From cultures of *Pseudomonas* sp. grown with *p*-cresol, Dagley & Patel (1957) prepared cell-free extracts that oxidized protocatechuic acid with an uptake of 1 mole of O_2 per mole of protocatechuate; β-oxoadipate was neither produced nor decomposed, and the end product of reaction was a dibasic acid which was not identified at that time. We have now shown that this compound is 2:4-lutidinic acid; the pyridine nucleus arises from a non-enzymic reaction between the initial product of fission of the benzene ring and ammonia present in the enzyme preparation. Determinations of equivalents by alkali titration, and analyses for C, H and N in the compound, were in excellent agreement with values calculated for the monohydrate, recrystallized from aqueous solution, and for the anhydrous acid obtained by sublimation under reduced pressure. Treatment with diazomethane gave a dimethyl ester with the expected molecular weight (Rast), m.p. and percentage composition. Reduction with platinum gave a hexahydro compound.

The enzymic reaction was re-investigated using well-dialysed extracts supplemented with mM-ferrous sulphate. For each mole of protocatechuic acid, 1 mole of O_2 was taken up, no CO_2 was evolved and an aldehyde accumulated which gave an acidic 2:4-dinitrophenylhydrazone. Aqueous solutions absorbed strongly at 410 mμ when alkaline, but this peak was abolished on acidification, with the appearance of a smaller peak at 290 mμ. Concentrated crude cell-free extracts contained enzymes that decomposed the compound, apparently to pyruvate. Evidently these extracts catalyse reactions similar to those described by Dagley & Stopher (1959) for catechol oxidation. Certain bacteria open the benzene ring between carbon atoms (C-1 and C-2) which carry hydroxyl groups and so produce a muconic acid (Evans, Smith, Linstead & Elvidge, 1951; Sistrom & Stanier, 1954), but in the present systems fission appears to occur between C-2 and C-3 to give a substituted muconic semialdehyde. The compound formed from catechol carries a hydroxyl group α to the carboxyl produced in the oxidation; that from protocatechuic acid also contains a second (γ) carboxyl group. In the presence of ammonia, ring closure between the terminal aldehyde and α-hydroxyl groups would give 2:4-lutidinic acid or α-picolinic acid, respectively, from the oxidation products of protocatechuate or catechol. Both of these non-enzymic reactions have been demonstrated, although the second is much slower.

We are indebted to Mr D. W. Ribbons for the proof that the formation of lutidinic acid is non-enzymic.

REFERENCES

Dagley, S. & Patel, M. D. (1957). *Biochem. J.* **66**, 227.
Dagley, S. & Stopher, D. A. (1959). *Biochem. J.* **73**, 16P.
Evans, W. C., Smith, B. S. W., Linstead, R. P. & Elvidge, J. A. (1951). *Nature, Lond.*, **168**, 772.
Sistrom, W. R. & Stanier, R. Y. (1954). *J. biol. Chem.* **210**, 821.

The Separation of Urine Colloids on Diethylaminoethylcellulose. By A. J. ANDERSON,* M. H. LEPPER and R. J. WINZLER. (*Departments of Biological Chemistry and Preventive Medicine, University of Illinois College of Medicine, Chicago, Illinois, U.S.A.*)

The colloids obtained by ultrafiltration of normal and pathological human urines have been fractionated on diethylaminoethylcellulose. The 'Varigrad' mixing device of Sober & Peterson (1958) was used, employing buffer mixtures of 2-amino-2-hydroxymethylpropane-1:3-diol and citrate, designed to produce an eluant with a gradually descending pH from 9 to 1·5, together with an increasing salt concentration. Analysis of the thirteen fractions obtained confirmed previous evidence (Maclagan & Anderson, 1958*a*) of the presence in urine colloids of proteins, glycoproteins containing sialic acid and fucose, and hexuronic acid-containing mucopolysaccharides.

In the fractions eluting above pH 6 immunoelectrophoretic evidence indicated the presence of at least seven components related to those in serum, including human-serum albumin and orosomucoid. The ratios hexose:hexosamine:sialic acid of these early fractions were similar to those reported by Winzler (1955) for the serum proteins.

Immunoelectrophoretic evidence indicated only traces of serum proteins in those fractions eluting below pH 6. These fractions contained larger amounts of hexuronic acid and were either immobile (paper electrophoresis, pH 8·6) or migrated with mobilities of α- and β-globulins. One of these fractions migrated considerably further at pH 4·6 than any serum component and may contain glycoproteins of low isoelectric point not present in serum.

The principal glycoprotein fraction of urine (Anderson & Maclagan, 1955) is known to inhibit viral haemagglutination (see Maclagan & Anderson, 1958*b*). It also contains an activator of plasminogen (Kickhöfen, Struwe, Bramesfeld & Westphal, 1958). Similar properties were noted in some of the fractions from the diethylaminoethylcellulose column.

The three fractions containing the greatest amount of sialic acid were found to be the most potent inhibitors of viral haemagglutination and these fractions reacted strongly with antibodies prepared against the 'uromucoid' fraction isolated by Boyce & Swanson (1955), the principal component of which is believed to be the strongly inhibitory glycoprotein isolated by Tamm & Horsfall (1952).

Activators of plasminogen were detected only in those fractions eluted from the column above pH 7, the greatest activities being associated with components of electrophoretic mobilities corresponding to γ- and β-globulins.

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REFERENCES

Anderson, A. J. & Maclagan, N. F. (1955). *Biochem. J.* **59**, 638.
Boyce, W. H. & Swanson, M. (1955). *J. clin. Invest.* **34**, 1581.
Kickhöfen, B., Struwe, F. E., Bramesfeld, B. & Westphal, O. (1958). *Biochem. Z.* **330**, 467.
Maclagan, N. F. & Anderson, A. J. (1958*a*). *Brit. J. Urol.* **30**, 269.
Maclagan, N. F. & Anderson, A. J. (1958*b*). In *Ciba Foundation Symposium on Chemistry and Biology of Mucopolysaccharides*, p. 268. London: Churchill and Co. Ltd.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 71.
Winzler, R. J. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 279. New York: Interscience Publ.

The Direct Chromatographic Estimation of Urinary Steroid Sulphates. By I. E. BUSH and M. M. GALE. (*Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford*)

The methods described previously for extracting (Bush & Gale, 1957) and separating (Bush, 1957) steroid conjugates were adequate for investigating steroid sulphates in blood and urine, but not sufficiently simple for a reliable routine method of estimation. The excellent performance of solvent systems based on *iso*propyl ether (Schneider & Lewbart, 1959) has, however, enabled us to work out a simple and reliable procedure.

A 100–200th part of a 24 hr. sample of urine is extracted with methylene chloride or ether–ethyl acetate (2:1, v/v) and the extract discarded. The urine is half-saturated with ammonium sulphate, made to N with concentrated ammonia solution, and extracted with ethyl acetate (2 × 3 vol.). The combined extracts are washed once with 7/8-saturated ammonium sulphate (1/20 vol.) and cleared by centrifuging and/or the addition of a minimal amount of filter paper. The extract is filtered, evaporated under reduced pressure, and

applied directly to the whole width of a 5 × 40–50 cm. strip of Whatman no. 2 paper: after 'running up' (Bush, 1952) with methanol–concentrated ammonia solution (19:1, v/v) the strip (with standards) is equilibrated for 3–16 hr. and run for 16–20 hr. in the system *iso*propyl ether–*tert.*-butanol–2N-ammonium hydroxide (2:1:3, v/v) at 18–26°. Complete separation is obtained of androsterone, aetiocholanolone and dehydro*epi*androsterone (DHA) sulphates, which can be estimated either by direct scanning (Bush & Mahesh, 1959) or by elution (Brooks, 1958) using alkaline *m*-dinitrobenzene.

This method is based on the highly favourable partition coefficients of the ammonium salts of steroid sulphates (Bush, 1957; Schneider & Lewbart, 1959) which makes their separation from glucuronosides by solvent partition easy.

The resulting chromatograms are remarkably clean and despite the use of a single chromatogram the method is likely to be sufficiently specific. Thus urines from patients and others on varying diets and drugs gave identical results for DHA sulphate measured by this method, and by a chromatographic modification of the method of Patterson & Swale (1953) in which DHA is estimated after hydrolysis at pH ⩾ 7·0.

Previous work suggested that only a small fraction of androsterone and aetiocholanolone was excreted as sulphate (Crepy, Jayle & Meslin, 1957; Kellie & Wade, 1957) and that practically all DHA was excreted as sulphate (Munson, Gallagher & Koch, 1944). The former results, however, were obtained by rather indirect methods. So far our results are similar except that the proportion of androsterone sulphate is occasionally much larger than expected from earlier work.

REFERENCES

Brooks, R. V. (1958). *Biochem. J.* **68**, 50.

Bush, I. E. (1952). *Biochem. J.* **50**, 370.

Bush, I. E. (1957). *Biochem. J.* **67**, 23P.

Bush, I. E. & Gale, M. M. (1957). *Biochem. J.* **67**, 29P.

Bush, I. E. & Mahesh, V. B. (1959). *J. Endocrin.* **18**, 1.

Crepy, O., Jayle, M. F. & Meslin, F. (1957). *Acta Endocrin.* **24**, 233.

Kellie, A. E. & Wade, A. P. (1957). *Biochem. J.* **66**, 196.

Munson, P. L., Gallagher, T. F. & Koch, F. C. (1944). *J. biol. Chem.* **152**, 67.

Patterson, J. & Swale, J. (1953). *Mem. Soc. Endocrin.* **2**, 41.

Schneider, J. J. & Lewbart, M. L. (1959). *Recent Progr. Hormone Res.* **15**, 201.

Effects of Salicylate and 2:4-Dinitrophenol on the Metabolism of [3-^{14}C]Pyruvate in Isolated Rat Tissues. By A. K. Huggins, V. Moses and M. J. H. Smith. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E.* 5)

Smith & Moses (1960) have shown that chopped preparations of isolated rat tissues incorporate measurable amounts of radioactivity from [^{14}C]-glucose and [2-^{14}C]acetate into soluble metabolic intermediates. The ^{14}C from both substrates was found to be incorporated into substances known to be involved in glycolysis, the hexose monophosphate oxidative pathway, the tricarboxylic acid cycle, transamination and synthetic reactions. However, the majority of radioactive intermediates found were concerned with the catabolism rather than the anabolism of the labelled substrates. Similar results have now been observed using [3-^{14}C]pyruvate. The incorporation of ^{14}C was restricted to amino and organic acids and no evidence of synthetic pathways was obtained. All the tissues (liver, kidney and brain) showed a similar pattern of incorporation of the radioactivity from the labelled pyruvate in the presence and absence of either 5 mM-salicylate or 0·5 mM-2:4-dinitrophenol.

A difference was observed between the effects of salicylate and 2:4-dinitrophenol on the distribution of ^{14}C between the amino and organic acid fractions. Salicylate produced proportionately less incorporation of radioactivity in the organic acids than in the amino acids whereas 2:4-dinitrophenol caused the reverse effect. The total incorporation of ^{14}C from the labelled pyruvate into the soluble intermediates of the tissue preparations was reduced by 20–30% in the presence of the uncoupling reagents. This inhibition of the total incorporation of ^{14}C was much less than that previously observed using [2-^{14}C]-acetate. The entry of the pyruvate carbon into the metabolic sequences of the tissues is therefore less dependent on the supply of adenosine triphosphate from oxidative phosphorylation processes than is the entry of the acetate carbon.

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REFERENCE

Smith, M. J. H. & Moses, V. (1960). *Biochem. J.* **74**, 17P.

A Clastic Fission of Pyruvic Acid in *Zymosarcina ventriculi*. By J. P. ARBUTHNOTT, T. BAUCHOP and E. A. DAWES. (*Department of Biochemistry, The University, Glasgow*)

We have shown previously that *Zymosarcina ventriculi* metabolizes pyruvate to yield acetaldehyde and CO_2 by a yeast-type carboxylase and we inferred also the presence of another pathway of pyruvate metabolism yielding formate (Bauchop & Dawes, 1959*a*, *b*). Earlier work was hampered by the fact that pyruvate was not appreciably metabolized by intact cells; we have now obtained cells active towards pyruvate by altering the conditions of growth.

An investigation of enzymic activity towards glucose, pyruvate and formate throughout the growth cycle revealed a steady decline in all these activities as growth progressed, and activity was lost completely after 3 hr. in the stationary phase. The loss of activity towards pyruvate can be attributed to permeability factors since it was shown previously that toluene-treated cells metabolized pyruvate. Furthermore, by partial digestion of the cell walls with 'suc digestif' from *Helix pomatia* and subsequent lysis, permeability has been found to be a limiting factor even with active cells.

Studies of pyruvate metabolism by intact cells at different pH values indicated that at pH 7·0 hydrogen is one of the main products but the ratio $CO_2:H_2$ varies and is always greater than unity. At pH 4·9 the ratio $CO_2:H_2$ is unity and the results can be explained in terms of a 'phosphoroclastic' fission of pyruvate to acetate and formate, followed by the metabolism of the latter to H_2 and CO_2 by the lyase system already shown to be present; one mole of pyruvate yields one mole each of volatile (acetic) acid, H_2 and CO_2. These results show that pyruvate is metabolized by both carboxylase and the clastic enzyme at pH 7·0, whereas at the lower pH only the clastic enzyme operates.

Cell-free extracts of the organism have been shown to contain hexokinase, aldolase and triosephosphate dehydrogenase, and to produce pyruvate from 3-phosphoglyceric acid; carboxylase is also present and the clastic enzyme has also been obtained, although it is extremely labile.

REFERENCES

Bauchop, T. & Dawes, E. A. (1959*a*). *Biochem. J.* **73**, 17P.
Bauchop, T. & Dawes, E. A. (1959*b*). *Biochim. biophys. Acta*, **36**, 294.

The Reactions of Bovine Meromyosins with Antiserum to Bovine Myosin. By I. G. S. FURMINGER (introduced by J. R. MARRACK). (*Department of Pathology and Low Temperature Research Station, University of Cambridge*)

Bovine myosin was extracted by pyrophosphate at pH 7·0 from longissimus dorsi muscle, stored 24 hr. at 0°, and an antiserum prepared towards it in a rabbit. The myosin was digested by trypsin (Lowey & Holtzer, 1959), to yield light and heavy meromyosins (Szent-Györgyi, 1953). An analysis of the cross-reactions of these proteins with the antiserum was carried out by double diffusion in agar, and the diffusion coefficients of the precipitate lines were measured according to Allison & Humphrey (1959).

In Allison & Humphrey plates the myosin preparation formed two lines of precipitate with anti-myosin serum, one corresponding to an antigen *a* having D^0 $3{\cdot}6 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, the other corresponding to an antigen *b* having D^0 $0{\cdot}95 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, the latter having a clear line on the antigen side. Clear lines have also been observed by Goudie, Anderson & Gray (1959) in certain anti-thyroglobulin sera. They cause a translucent line in the opaque agar which is not removed when the excess antigen and antibody is washed out before staining, and themselves stain like a normal precipitate. The light meromyosin preparation also formed two lines, one corresponding to an antigen *a* having D^0 $3{\cdot}5 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, and a clear line corresponding to an antigen *b* having D^0 $0{\cdot}95 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ The heavy meromyosin formed only one line, corresponding to an antigen having D^0 $3{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ The best values for the diffusion coefficients of myosin, light and heavy meromyosins are, respectively, 1·05 (Parrish & Mommaerts, 1954), 2·87 and 2·91 (Szent-Györgyi, 1953).

In Ouchterlony plates, lines *a* formed by myosin with antimyosin was continuous with that of *a* formed by the light meromyosin, and *b* formed by the light meromyosin was continuous with the clear line formed by myosin. The single line of the heavy meromyosin was continuous with *b* of myosin, but the latter formed a spur.

Bovine light meromyosin, unlike the pig light meromyosin, did not crystallize, and was found to contain 5–10% of a heavier component, presumably myosin, on ultracentrifugation. This heavy component could possibly account for precipitate *b* formed in Ouchterlony plates. One may, therefore, conclude that the light meromyosin is

responsible for precipitate *a* and possibly the clear line, whereas the fraction in myosin that forms the heavy meromyosin when digested, forms precipitate *b*. Line *a* formed by myosin and line *a* formed by the light meromyosin are from the same antigen. Therefore the light meromyosin in our preparation appears to be a separate protein, as suggested by Holtzer & Marshall (1958). This, however, is contrary to most current opinions and its presence in the myosin preparation could be explained by a breakdown due to cathepsins during the period between slaughter and extraction.

REFERENCES

Allison, A. C. & Humphrey, J. H. (1959). *Nature, Lond.*, **183**, 1590.
Goudie, R. B., Anderson, J. R. & Gray, K. G. (1959). *Immunology*, **2**, 309.
Holtzer, M. & Marshall, J. M. (1958). *Biophys. Soc. Meeting*. Boston, U.S.A.
Lowey, S. & Holtzer, A. (1959). *Biochim. biophys. Acta*, **34**, 470.
Parrish, R. G. & Mommaerts, W. F. H. M. (1954). *J. biol. Chem.* **209**, 901.
Szent-Györgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.

Serological Classification of Rabbit γ-Globulins. By A. Kelus (introduced by J. R. Marrack). (*Department of Pathology, University of Cambridge*)

The γ-globulin fraction, as the part of human and animal serum carrying most antibodies, is of great interest for immunochemists. Like other protein it can also be considered as an antigenic mixture. Recently the cross-immunization of individual rabbits with serum protein of the same species (*Oryctolagus cuniculus*) was accomplished (Oudin, 1956; Dray & Young, 1958; Dubiski, Dudziak, Skalba & Dubiska, 1959), and it was shown that the γ-globulins have a complex antigenic structure (Dubiski, Skalba, Dubiska & Kelus, 1959).

Bacteria coated with homologous rabbit antibodies were injected into rabbits which produced iso-precipitins of various specificity reacting with some other rabbit sera used as antigens. These antigens, analysed by immunoelectrophoresis, have the mobility of γ-globulin. It is possible to identify at least five antigens designated as A, B, C, D and E. They form 22 groups, out of 32 theoretically possible, and are differently distributed in separate rabbit populations. It is remarkable that some random-mating strains of rabbits possess only one or two antigens.

The γ-globulin antigens described above are of hereditary character and they seem to belong to three genetic systems (A, C and BDE), statistically independent.

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REFERENCES

Dray, S. & Young, G. P. (1958). *J. Immunol.* **81**, 142.
Dubiski, S., Dudziak, Z., Skalba, D. & Dubiska, A. (1959). *Immunology*, **2**, 84.
Dubiski, S., Skalba, D., Dubiska, A. & Kelus, A. (1959). *Nature, Lond.*, **184**, 1811.
Oudin, M. J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2489, 2606.

Specificity of the Antigens of Rabbit γ-Globulin, Hydrolysed by Papain. By A. Kelus, J. R. Marrack and C. B. Richards. (*Departments of Pathology and Colloid Science, University of Cambridge*)

Rabbit γ-globulin, group aBcdE (Dubiski, Skalba, Dubiska & Kelus, 1959), was hydrolysed with crystalline papain and fractionated on carboxymethylcellulose (Porter, 1959). Examination of the original γ-globulin and the fractions in the Spinco analytical ultracentrifuge gave results in accordance with those previously obtained (Charlwood, 1959).

The crystalline fraction formed a precipitate with goat anti-rabbit serum. It did not react with rabbit anti-B–γ-globulin serum, whereas fractions 1 and 2 did. The crystalline fraction added to goat anti-rabbit serum inhibited formation of a precipitate with the fastest (highest electrophoretical mobility) antigenic γ-globulin of rabbit serum, but did not inhibit precipitation with other globulins whose mobilities overlapped those of γ-globulin. This suggests that the species specific antigens and the isospecific antigen B are located on different pieces of the γ-globulin molecule.

Similar investigations on other rabbit γ-globulins of various antigenic structure are in progress.

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REFERENCES

Charlwood, P. A. (1959). *Biochem. J.* **73**, 126.
Dubiski, S., Skalba, D., Dubiska, A. & Kelus, A. (1959). *Nature, Lond.*, **184**, 1811.
Porter, R. R. (1959). *Biochem. J.* **73**, 119.

The Extraction of Lipid Matter by Formalin. By J. CHAYEN, R. CHAYEN and E. K. AVES. (*Department of Pathology, Royal College of Surgeons, Lincoln's Inn Fields, London, W.C. 2, and Department of Chemical Engineering, University College, London, W.C. 1*)

It has been shown, by histochemical procedures, that formalin fixation removes lipid and protein components from the heterochromatic regions of interphase nuclei (Chayen, Gahan & La Cour, 1959), and that this complex is capable of concentrating the carcinogen, 3:4-benzpyrene, from an hydrotropic solution (Chayen, Gahan & La Cour, 1957). Possibly the carcinogenicity of compounds such as benzpyrene, is related to whether or not they are taken up by the nucleus (Calcutt, 1958), so that it seemed that this formalin-soluble complex might be the site of action of such carcinogenic agents. However, the only chemical evidence concerning the lipid nature of the extracted material was its solubility when removed from the formalin and the demonstration, after suitable hydrolysis, that it contained glycerophosphate.

Large quantities have now been obtained of the formalin extract from human lung. The dried extract, which contained 1 g. of acid-precipitable material, was first heated in boiling 0·5N-potassium hydroxide in 95% ethanol for 3 hr. This yielded 120 mg. of non-saponifiable matter; in the saponified fraction, i.e. ether-extracted after acidification, only a single unidentified acidic substance has been detected by the method of reversed-phase paper chromatography of Chayen & Linday (1960). The potassium hydroxide hydrolysis left a considerable residue which was then heated in boiling 6N-hydrochloric acid under reflux for 5 hr. to give a clear solution. On extraction with ether, a wide range of fatty acids was obtained; these were shown, by vapour-phase chromatography of the methyl esters, to include mainly palmitic acid, but also considerable amounts of stearic and probably oleic, and small amounts of myristic, lauric and other acids. Glycerophosphate and many amino acids were demonstrated by paper chromatographic analysis of the aqueous phase.

Thus it has been shown that the formalin, used as a fixative, has extracted material which is truly lipid in that it contains fatty acids, so confirming the histochemical data.

REFERENCES

Calcutt, G. (1958). *Brit. J. Cancer*, **12**, 149.
Chayen, J., Gahan, P. B. & La Cour, L. F. (1957). *Nature, Lond.*, **180**, 652.
Chayen, J., Gahan, P. B. & La Cour, L. F. (1959). *Quart. J. micr. Sci.* **100**, 279.
Chayen, R. & Linday, E. M. (1960). *J. Chromat.* (in the Press).

The Fate of Physiological Doses of ^{14}C-Labelled Vitamin D_2 in Normal and Rachitic Rats. By E. KODICEK and D. R. ASHBY. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

In previous experiments doses ranging from 0·25 to 1 mg. (40 000 i.u.) of vitamin D_2 were given to young rats (Kodicek, 1954, 1956). There was a valid objection that non-physiological doses were employed and that the distribution of the vitamin in the tissues was due to the excessive amounts administered. Therefore, a single dose of only 10 μg. (400 i.u.) of uniformly ^{14}C-labelled vitamin D_2 (specific activity 1·15 μc/mg.) was given by stomach tube to a group of rachitic and of normal rats, fed on Steenbock diet no. 2965; the normal group received adequate vitamin D_2 (30 i.u./rat/day). Two other groups of rachitic and normal rats were given 1 mg. of ^{14}C-labelled vitamin D_2. After 24 hr., tissues and faeces of the rats were analysed for ^{14}C-labelled vitamin D and breakdown products by fractionation techniques (Kodicek, 1956) and by reversed-phase paper chromatography (Kodicek & Ashby, 1954).

The recovery of total ^{14}C expressed as percentage of the dose of 10 μg., in rachitic and normal rats, respectively, was as follows (the figures in brackets denote vitamin D): in faeces and gut content, 59·4, 38·3 (31·0, 26·0); in liver, 8·5, 7·6 (5·8, 7·3); in intestinal tissue, 2·0, 0·5 (0·8, 0·5); in bones, 0·4, 0·7 (0·1, 0·3); in the rest of the body, 1·0 and 2·4 (1·0, 2·4).

The distribution of ^{14}C in rats given 1 mg. of ^{14}C-labelled vitamin D_2 was similar: in faeces and gut contents, 51·5, 18·0 (33·5, 7·5); in liver, 7·4, 7·6 (6·0, 5·6); in intestinal tissue, 0·4, 0·7 (0·3, 0·4); in bones, 0·8, 0·9 (0·6, 0·8); in the rest of the body, 2·6 and 21·0 (2·6, 17·8).

The distribution of ^{14}C in the tissues of animals given 10 μg. of ^{14}C-labelled vitamin D_2 was similar to that obtained with 1 mg. of vitamin; 40–60% of the dose was in the faeces. No difference in the deposition or excretion of vitamin D was observed between the rachitic and normal animals. This finding differs from previous results (Kodicek &

Ashby, 1960; Blumberg, Schönholzer & Aebi, 1959) where less ^{14}C-labelled vitamin D appeared to accumulate in bones and other tissues of normal stock rats. In the present experiments, the high-calcium, low-phosphorus diet may have provided an additional stress on the vitamin D metabolism and consequently resulted in a higher turnover of the vitamin. The vitamin content of the liver of rachitic and normal rats, given the 10 μg. dose, was of the same order of magnitude as that of rachitic rats irradiated with ultraviolet light (Cruickshank & Kodicek, 1955), indicating that the dose was within the physiological range.

The findings in rats given 1 mg. of ^{14}C-labelled vitamin D_2 compared well with previous experiments, with the exception of a relatively low recovery in the faeces of normal animals.

REFERENCES

Blumberg, A., Schönholzer, G. & Aebi, H. (1959). *Helv. physiol. acta*, **17**, C16.

Cruickshank, E. M. & Kodicek, E. (1955). *Proc. Nutr. Soc.* **14**, viii.

Kodicek, E. (1954). *Biochem. J.* **58**, xxxvi.

Kodicek, E. (1956). In *Ciba Foundation Symposium on Bone Structure and Metabolism*, p. 161. London: Churchill and Co. Ltd.

Kodicek, E. & Ashby, D. R. (1954). *Biochem. J.* **57**, xiii.

Kodicek, E. & Ashby, D. R. (1960). *Biochem. J.* (in the Press).

The Metabolism of ^{14}C-Labelled Vitamin D_2 Injected Intracardially into Rats. By E. Kodicek, E. M. Cruickshank and D. R. Ashby. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

At 48 hr. after a peroral dose of 1 mg. of vitamin D_2, the adrenals contain a concentration of vitamin D equal to that of liver (Cruickshank, Kodicek & Armitage, 1954). ^{14}C-Labelled vitamin D was found by radioautographs mainly in the zona fasciculata of rat adrenals (Kodicek, 1960). Raoul & Gounelle (1958) recently reported that 90% of intravenously injected vitamin D_3 was, during the first 30 min., deposited in the adrenals of rats, and in liver only 2 hr. after injection. Their findings would indicate an important involvement of adrenals in the metabolism of vitamin D.

A dose of 50 μg. of uniformly ^{14}C-labelled vitamin D_2 (specific activity 0·1 μc/mg.), was dissolved in 10 μl. of ethanol, mixed with 0·1 ml. of rat serum and injected intracardially into each of six stock rats, weighing 170–190 g. The rats were killed, in groups of two, 20 min., 40 min. and 5 hr. after injection. The tissues and faeces were analysed for ^{14}C by fractionation techniques (Kodicek, 1956) and by reversed-phase paper chromatography (Kodicek & Ashby, 1954). In a second experiment two rats were injected with 200 μg. of ^{14}C-labelled vitamin D_2 and the tissues analysed after 20 min. and 5 hr.

The adrenals contained after 20 min. only 1·3% of the ^{14}C-dose (1% as vitamin D); after 40 min. 0·2%; after 5 hr. 0·5% (0·2% as vitamin D). Liver accumulated the major part of the ^{14}C; after 20 min., 54·3% (48·9% as vitamin D); after 40 min., 60·1% (55·4% as vitamin D); after 5 hr., 50% (47% as vitamin D). Blood carried 10·7% of ^{14}C in 20 min., 11·3% in 40 min., and 9·1% in 5 hr., mainly as vitamin D bound to serum proteins (Innes Chalk & Kodicek, 1960). The kidneys had 0·3, 3·9 and 2·3% of ^{14}C; intestinal tissue had 1·5, 0·4 and 1·3% at 20 min., 40 min. and 5 hr., respectively. In the first 20 min., 1·2% of ^{14}C appeared in the intestinal contents, after 5 hr. 2·5%, mainly in the lower parts of the intestinal tract and in faeces. Similar results were obtained by injecting 50 μg. of vitamin D_3 labelled with ^{14}C at C-4.

In our experimental conditions, the bulk of the dose (50–60%) accumulated in the liver as early as 20 min. after injection, but very little appeared in the adrenals. A fairly large proportion of the dose circulated in the blood for 5 hr. The amount of ^{14}C-labelled vitamin D was greater in the upper than in the lower part of the intestinal tissue, a fact which may be of significance with regard to the absorption of calcium, which appears to occur in the duodenum and jejunum (Rasmussen, 1959; Schachter & Rosen, 1959). The distribution differed from that found in rats given vitamin D_2 intramuscularly, where the lower parts of the intestinal tissue were richer in the vitamin (Cruickshank & Kodicek, 1958).

REFERENCES

Cruickshank, E. M. & Kodicek, E. (1958). *Abstr. IVth int. Congr. Biochem., Vienna*, p. 90.

Cruickshank, E. M., Kodicek, E. & Armitage, P. (1954). *Biochem. J.* **58**, 172.

Innes Chalk, K. J. & Kodicek, E. (1960). *Biochem. J.* **74**, 22P.

Kodicek, E. (1956). In *Ciba Foundation Symposium on Bone Structure and Metabolism*, p. 161. London: Churchill and Co. Ltd.

Kodicek, E. (1960). In *Vitamin Metabolism*, p. 198. London: Pergamon Press.

Kodicek, E. & Ashby, D. R. (1954). *Biochem. J.* **57**, xiii.

Raoul, Y. & Gounelle, J.-C. (1958). *C.R. Acad. Sci., Paris*, **247**, 161.

Rasmussen, H. (1959). *Endocrinology*, **65**, 517.

Schachter, D. & Rosen, S. M. (1959). *Amer. J. Physiol.* **196**, 357.

Characterization of Constituents of Blood Plasma and of the Brain which Restore Excitability to Isolated Cerebral Tissues. By H. McIlwain. (*Department of Biochemistry, Institute of Psychiatry, London, S.E.* 5)

Cerebral tissues kept in cold media lose their respiratory and other responses to applied electrical pulses. Excitability can be restored by added materials, including fractions of blood-plasma proteins and extracts from the brain (Marks & McIlwain, 1959). A large number of known substances are inactive in this regard.

Qualitative tests on the active plasma fractions have now shown them to contain carbohydrate and sialic acid derivatives; these with other properties are indicative of mucoproteins. Neuraminic acid has therefore been determined according to Bohm, Dauber & Baumeister (1954), and ability to restore excitability has been found to be correlated with neuraminic acid content. The following values were obtained with ox-plasma fractions, expressing *N*-acetylneuraminic acid content in mg./g. of dry matter, and restoration of excitability as the percentage increase in respiratory rate (above control without addition) caused by addition of 10 mg. of plasma fraction/ml. of medium. Original plasma; 6 mg., 14%. Fractions of similar activity; III, 1: 6·6 mg., 12%; III, 2: 6·8 mg., 10%. Less active fractions; I: 4 mg., 5%; II: 2·4 mg., 7%. Enriched fractions; IV, 1: 19 mg., 33%; IV, 4: 22 mg., 51%.

The main neuraminic acid derivatives of the brain are the gangliosides, and in the knowledge that Van Heyningen (1959) had shown gangliosides to be the probable point of action of tetanus toxin on the central nervous system, ganglioside preparations have been examined for their ability to restore excitability to the treated cerebral tissues. Three independent preparations, made according to Folch, Arsove & Meath (1951) and Rosenberg & Chargaff (1958) were active in concentrations of 0·1 mg./ml. This involved the addition of a quantity of gangliosides approximating to that normally present in the tissue sample examined.

The gangliosides and the neuraminic acid-containing mucoproteins are large molecules with certain but not all components in common. Several of the constituents are already known to be incapable of restoring excitability to the treated cerebral tissues, and further investigation in this regard is in progress. As the excitability and the main functional events in cerebral tissues are concerned with ion movements, it appears significant that in other cells, the neuraminic acid of large molecules is associated with the maintenance of their surface charge (see Gottschalk & Klenk, 1958).

The author is greatly indebted to Dr C. Long for a ganglioside preparation, to Miss P. Carroll for synthetic *N*-acetylneuraminic acid and to Dr Long and Dr W. E. van Heyningen for discussion.

REFERENCES

Bohm, P., Dauber, St. & Baumeister, L. (1954). *Klin. Wschr.* **32**, 289.

Folch, J., Arsove, S. & Meath, J. A. (1951). *J. biol. Chem.* **191**, 819.

Gottschalk, A. & Klenk, E. (1958). In *Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides*, p. 311. London: Churchill and Co. Ltd.

Marks, N. & McIlwain, H. (1959). *Biochem. J.* **73**, 401.

Rosenberg, A. & Chargaff, E. (1958). *J. biol. Chem.* **232**, 1031.

Van Heyningen, W. E. (1959). *J. gen. Microbiol.* **20**, 310.

The Structure of Bradykinin. By D. F. Elliott, G. P. Lewis and E. W. Horton. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Bradykinin, isolated from ox blood, was found to have the amino acid composition Ser(1):Gly(1):Pro(2):Phe(2):Arg(2) and a single *N*-terminal residue of arginine (Elliott, Lewis & Horton, 1960). The structure has now been elucidated by chemical and enzymic degradation. Chymotrypsin hydrolysed two peptide bonds in the molecule, one bond being more rapidly split than the other. The main hydrolysis products were arginine and a heptapeptide; the latter was shown to possess a *C*-terminal phenylalanine residue by the use of carboxypeptidase. At a higher concentration of chymotrypsin, appreciable hydrolysis of the second phenylalanyl bond took place yielding, as one of the products, a pentapeptide containing arginine, proline, glycine and phenylalanine and as another a peptide yielding serine and phenylalanine on hydrolysis. Stepwise degradation of bradykinin by the Edman method indicated the sequence Arg.Pro.Pro.Gly.Phe for the first five residues. Therefore the structure of bradykinin is Arg.Pro.Pro.Gly.Phe.Ser.Phe.Arg.

REFERENCE

Elliott, D. F., Lewis, G. P. & Horton, E. W. (1960). *Biochem. J.* **74**, 15P.

Non-Enzymic Interaction of Phosphate and Glucose in Aqueous Solutions. By S. LEWIN. (*Physical Biochemistry Research School, South-West Essex Technical College, London, E.* 17)

The acceleration of the breakdown of reducing sugars, and the associated development of brown colour, in presence of buffers such as phosphates has been noted by several investigators (for references, see Hodge, 1953). The findings that amino acid–reducing sugar solutions give rise to a series of steady pH states (Lewin, 1956*a*, *b*; Lewin & Fox, 1956; Lewin & Kosinski, 1958; Lewin & McCall, 1957) accompanied by the development of brown colour at the end of the second steady pH state or the beginning of the third steady pH state suggested the possibility that the mechanism by which phosphate affects reducing sugars may be associated with a similar phenomenon.

It has already been reported (Lewin, 1957) that during 30 hr., at 40° the pH of the mixture remained constant and only glucose could be chromatographically detected. An extended study of such mixtures revealed that the original short-term results were reproducible, but that subsequently a series of steady pH states were detectable at both 30° and 40°. Paper-chromatographic techniques failed to reveal any other sugar besides glucose in the beginning, but after eight days at 40° fructose was indicated. With the passage of time fructose formation became more pronounced. Comparison of ascending and simplified arc paper-chromatographic techniques (Lewin, 1959) showed the latter method to be more sensitive in that its fructose indication was clearer.

REFERENCES

Hodge, J. E. (1953). *Agric. Food Chem.* **1**, 928.
Lewin, S. (1956*a*). *Biochem. J.* **63**, 14.
Lewin, S. (1956*b*). *Biochem. J.* **63**, 5P.
Lewin, S. (1957). *Biochem. J.* **65**, 30P.
Lewin, S. (1959). *Lab. Practice*, **8**, 84.
Lewin, S. & Fox, C. (1956). *Biochem. J.* **63**, 5P.
Lewin, S. & Kosinski, Z. (1958). *Trans. Faraday Soc.* **54**, 222.
Lewin, S. & McCall, J. (1957). *Biochem. J.* **65**, 30P.

Mechanism of the Reduction of Methylene Blue in Glucose–Glycine–Phosphate Solutions. By S. LEWIN. (*Physical Biochemistry Research School, South-West Essex Technical College, London, E.* 17)

Several investigators, including Borsook & Wasteneys (1925), Euler, Brunius & Josephson (1928), and Lieben & Getreuer (1932), observed that amino acid–glucose mixtures in phosphate solutions were capable of reducing methylene blue at 37° and at higher temperatures in neutral and slightly acid solutions, where glucose itself was unable to do so. It was suggested that this reduction is due to a compound formed between the amino acid and the sugar. The formation of such compounds, on the basis of losses of free amino groups in the amino acid–sugar mixtures, has also been advocated by other investigators [see Danehy & Pigman (1951), Hodge (1953)]. Using potentiometric methods, Katchalsky (1941) and Frankel & Katchalsky (1941) interpreted their results in terms of interaction of one molecule of glucose with one molecule of the anion of the amino acid, while Lewin *et al.* (1956, 1957, 1958), interpreted the results obtained by them for certain amino acids in the period of the first steady pH state in terms of co-existence of mono- and di-glucose interactions with the amino acid anion.

We have examined, colorimetrically, the kinetics of the reduction of methylene blue in glycine–glucose–phosphate solutions (0·1–0·2M-glycine, 0·1–0·5M-glucose), at 37° under nitrogen free from oxygen, over the pH range 6–8 in the period of the first steady pH state. The reduction was found to be first order with respect to the methylene blue. The velocity constant of the reduction was found to be almost directly proportional to the pH and also to the glucose concentration over the lower concentrations. The findings of the present investigation may be co-related with the findings of Lewin & Kosinski (1958) on the assumption that the reduction is primarily due to the amino acid anion–monoglucose complex, the concentration of which is predominant at low glucose concentrations. However, it is possible that reductions due to phosphate associations participate. This possibility is supported by the observations (for references, see Hodge, 1953) confirmed in this laboratory that phosphate accelerates the development of the browning reaction and by other findings by the author of definite interaction between phosphate and glucose in phosphate–glucose solutions (Lewin, 1960).

REFERENCES

Borsook, H. & Wasteneys, H. (1925). *Biochem. J.* **19**, 1128.

Danehy, J. P. & Pigman, W. W. (1951). *Advanc. Food Res.* **3**, 241.

Euler, H., Brunius, E. & Josephson, K. (1928). *Svensk kem. Tidskr.* **40**, 163.

Frankel, M. & Katchalsky, A. (1941). *Biochem. J.* **35**, 1028.

Hodge, J. E. (1953). *J. agric. Food Chem.* **1**, 928.

Katchalsky, A. (1941). *Biochem. J.* **35**, 1024.

Lewin, S. (1956). *Biochem. J.* **63**, 14.

Lewin, S. (1960). *Biochem. J.* **76**, 17P.

Lewin, S. & Fox, C. (1956). *Biochem. J.* **63**, 5P.

Lewin, S. & Kosinski, Z. (1958). *Trans. Faraday Soc.* **54**, 222.

Lewin, S. & McCall, J. S. (1957). *Biochem. J.* **65**, 30P.

Lieben, F. & Getreuer, V. (1932). *Biochem. Z.* **252**, 420.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **394th Meeting** *of the Society was held in conjunction with the Société de Chimie Biologique in the Faculté de Pharmacie, Avenue de l'Observatoire, Paris 6e, on Friday and Saturday, 27, 28 May* 1960, *when the following papers were read by Members of The Biochemical Society.*

* Indicates that the Member received a grant from the Travel Fund of the Society.

COMMUNICATIONS

Transport of Iodide by the Small Intestine of the Rat *in vitro*.

By J. D. Acland and Sheila Johnson. (*Department of Pharmacology and Therapeutics, Sheffield University, Sheffield* 10)

The central region of the small intestine of the rat has been shown to transport I^- against a concentration gradient ('actively') from its serosal to its mucosal side *in vivo* (Pastan, 1957) and in aerobic conditions *in vitro* (Acland & Illman, 1959). In the present investigation transport of I^- by the small intestine has been studied in groups of male albino rats given the following treatments: (*a*) a diet low in I (containing about 50 μg. of I/kg.) for at least 4 weeks (8 animals); (*b*) a normal diet with the addition of 0·02% by weight of the antithyroid substance 2-ethoxycarbonylthio-1-methylglyoxaline (carbimazole, B.P.), to their drinking water (8 animals) for 4 weeks; (*c*) a normal diet with carbimazole for 4 weeks, followed by a diet low in I, with carbimazole, for another 4 weeks (3 animals); (*d*) a normal diet with intramuscular injections of 5 mg. of KI three times a week (4 animals).

Rates of transport of I^- were measured by the everted sac technique of Wilson & Wiseman (1954) as previously described (Acland & Illman, 1959), using 0·1 mM-$K^{131}I$ (about 1 μC/ml.) in Krebs–Henseleit bicarbonate buffer. In the case of the experiments on carbimazole-treated rats the buffer also contained carbimazole (0·114 mM).

The maximum rates of transport of I^- in all groups did not differ significantly from the values observed by Acland & Illman (unpublished data) in 11 normal animals (mean 0·38 μm-equiv./mg. wet wt./hr.; range 0·12–0·95).

These results confirm that the mechanism of active transport of I^- in the rat jejunum does not involve a peroxidase-like oxidative reaction (Acland & Illman, 1959) and suggest that it is also unaffected by the level of circulating thyroid-stimulating hormone.

REFERENCES

Acland, J. D. & Illman, O. (1959). *J. Physiol.* **147**, 260.
Pastan, I. (1957). *Endocrinology*, **61**, 93.
Wilson, T. H. & Wiseman, G. (1954). *J. Physiol.* **123**, 116.

Interrelation of Calcium and Magnesium Absorption.

By N. Alcock* and I. MacIntyre. (*Department of Chemical Pathology, Postgraduate Medical School, London, W.* 12)

Hooded rats of initial weight 90–110 g. were fed either a calcium-deficient or magnesium-deficient diet. Faecal and urinary excretion of calcium and magnesium was compared with that of a control group of rats which received calcium and magnesium in normal amounts.

A technique for conducting balance studies in the rat was used (Hanna & Alcock, unpublished). A synthetic diet, prepared in liquid form, was quantitatively fed by stomach tube. During balance periods rats were placed individually in Perspex metabolic cages. A constant amount of chromium sesquioxide was added to the diet. This substance is not absorbed from the gut and if its concentration in the faeces is known then the total faecal excretion of calcium and magnesium can be calculated.

Results showed that in the magnesium-deficient rat the faecal excretion of calcium was significantly lower ($P < 0·01$, analysis of variance) than that in rats receiving normal amounts of calcium and magnesium. The mean (no.) $\pm$ S.E.M. in the experimental group was 1563 (61) $\pm$ 56·0 μequiv./24 hr., compared with 1735 (67) $\pm$ 57·0 μequiv./24 hr. in the control group. There was no difference between the concentration of chromium sesquioxide in the two groups. Conversely, in the absence of calcium from the diet, less magnesium [84·9 (26) $\pm$ 6·4 μequiv./24 hr.] was excreted than normal [154·0 (28) $\pm$ 6·28 μequiv./24 hr.].

The net absorption of calcium in the magnesium-deficient animal was equivalent to the total amount of calcium and magnesium absorbed in the normal group.

It was also shown that from the first day of magnesium deficiency, urinary excretion of calcium was diminished, despite the presence of hypercalcaemia. No change in the glomerular filtration rate was detected and the decreased urinary excretion of calcium may have been due to an increase in absorption of filtered calcium by the renal tubular cells.

Experiments *in vitro* in the rat, rabbit and guinea pig have shown that calcium ions can be actively transported from the mucosal to the serosal surfaces of everted gut sacs, prepared from segments of the proximal end of the small intestine (Schachter & Rosen, 1959). Transport of calcium was depressed in the presence of magnesium ions, suggesting competition between the two.

The work presented here, together with that carried out *in vitro*, is in agreement with that of Berglund & Forster (1957) who suggest a common transport mechanism for calcium and magnesium.

REFERENCES

Berglund, F. & Forster, R. P. (1957). *J. gen. Physiol.* **41**, 429.

Schachter, D. & Rosen, S. M. (1959). *Amer. J. Physiol.* **196**, 357.

The Cytidine Diphosphate Choline Content of Brain Tissue.

By G. B. Ansell* and Brenda J. Bayliss. (*Department of Experimental Psychiatry, The Medical School, Birmingham* 15)

It is known from work with cell-free preparations that there is an absolute requirement for cytidine triphosphate in the synthesis of brain lecithin (McMurray, Strickland, Berry & Rossiter, 1957). The resolution of cerebral nucleotides on Dowex 1 (formate) has shown, however, that the cytidine components do not account for more than about 3% of the total nucleotides present in an acid-soluble extract of the tissue (Mandel & Harth, 1957). As a result of a more elaborate analysis, Koransky (1958) concluded that the cytidine nucleotide content was negligible and commented that this was surprising in view of the capacity of cerebral tissues to synthesize phospholipids. Subsequently, Koransky (personal communication) has stated that low concentrations of cytidine nucleotides are present.

It seemed desirable to confirm the presence of cytidine diphosphate choline in brain tissue, and the following relatively simple method of fractionation has been devised. Brain tissue from rats frozen whole in liquid nitrogen was rapidly extracted with 10% trichloroacetic acid solution and, after removal of this acid with ether, the pH of the extract was brought to 8·5–9·0 with NH_4OH. In some experiments extracts were prepared with 66% ethanol as described by Kennedy & Weiss (1956). Nucleotides were adsorbed on a column of Dowex 1 (chloride) and eluted with 0·1 M-NH_4OH–0·1 M-NH_4Cl, which had been shown to elute added cytidine diphosphate choline quantitatively. The nucleotide fraction was adsorbed from acid solution on to a column of Norit SX Plus–Celite 535 (1:5, w/w) (cf. Threfall, 1957) and eluted with 50% ethanol containing 0·33% (w/v) NH_3.

Two-dimensional paper chromatography of this eluate in ethanol–0·05 N-acetic acid (3:2, v/v) and 0·2 M-ammonium acetate–0·021 M-ethylenediaminetetra-acetic acid–95% ethanol (1:1:5, v/v) showed the presence of at least six nucleotides, of which one behaved in all respects like cytidine diphosphate choline. Hydrolysis and other studies showed that it was uncontaminated with the corresponding ethanolamine nucleotide.

A minimum of 5 g. of brain tissue was necessary to demonstrate the compound and recoveries of synthetic material added at the beginning of the separation amounted to 60%. Further experiments, carried out on extracts from brains heavily labelled *in vivo* with ^{32}P by subarachnoid injection (Lindberg & Ernster, 1950) showed that the amount present in brain, calculated on the basis of isotope dilution, agreed well with the amount calculated from recovery experiments. It is estimated that the amount of cytidine diphosphate choline present in 3–4-week-old rat brain is 0·02–0·03 μmole/g. fresh weight.

REFERENCES

Kennedy, E. P. & Weiss, S. B. (1956). *J. biol. Chem.* **222**, 193.

Koransky, W. (1958). *Arch. exp. Path. Pharmak.* **234**, 46.

Lindberg, O. & Ernster, L. (1950). *Biochem. J.* **46**, 43.

McMurray, W. C., Strickland, K. P., Berry, J. F. & Rossiter, R. J. (1957). *Biochem. J.* **66**, 634.

Mandel, P. & Harth, S. (1957). *C.R. Acad. Sci., Paris*, **245**, 1843.

Threlfall, C. J. (1957). *Biochem. J.* **65**, 694.

The Oxygen Consumption of Circulating Human Leucocytes after Intravenous Injection of Tri-iodothyroacetic Acid and Tri-iodothyronine.

By Sheenah K. Bisset, W. D. Alexander and J. Crooks. (*Department of Biochemistry and Gardiner Institute of Medicine, Western Infirmary, Glasgow*)

Two patients with untreated myxoedema, but without any evidence of coronary disease, were chosen as suitable subjects for this investigation.

The first patient, after resting and fasting for 12 hr., was given an intravenous injection of 5 mg. of tri-iodothyroacetic acid (TRIAC) in 10 ml. of isotonic saline. Samples of blood were taken at −30, +30, +60, +120 and +180 min. before and after injection. The method of separation of leucocytes for investigation has been described

previously (Bisset & Alexander, 1960). The rate of oxygen uptake of the leucocytes was determined in Warburg flasks at 37°. The total metabolic rate of the patient was determined at −30, +60, +120 and +390 min. before and after injection. The rate of oxygen consumption of the patient's leucocytes increased by 217% at +180 min. The total metabolic rate of the patient increased by 42% at 390 min. When this patient was given an injection of 10 ml. of isotonic saline, the oxygen consumption of the leucocytes and the total metabolic rate of the patient remained at the initial values.

The second patient was given an intravenous injection of 5 mg. of TRIAC and 7 days later, when the metabolic rate had returned to the initial level, the patient was given an intravenous injection of 2 mg. of tri-iodothyronine. With the TRIAC injection, the maximal increase in the rate of oxygen consumption of the circulating leucocytes occurred at 1 hr. and amounted to 55% over the control value. The maximal response in total metabolic rate was an increase of 16%. When the 2 mg. of tri-iodothyronine was given to this patient the maximal increase in the rate of oxygen consumption of the leucocytes took place 2 hr. after injection and measured 52% over the control value. No increase was found in the recorded metabolic rates of the patient.

It has already been demonstrated by us that the rate of oxygen consumption of circulating leucocytes is directly related to the basal metabolic rate of subjects in the hyperthyroid, euthyroid and hypothyroid states (Alexander & Bisset, 1958).

From these results, we conclude that the respiration of circulating human leucocytes can be rapidly influenced by the hormones TRIAC and tri-iodothyronine.

REFERENCES

Alexander, W. D. & Bisset, S. K. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, pp. 9–22.

Bisset, S. K. & Alexander, W. D. (1960). *Quart. J. exp. Physiol.* **45**, 18.

The Action of γ-Rays on Collagen.

By J. H. Bowes, J. A. Moss and A. S. Raistrick. (*British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey*)

Most of the work so far reported on the action of ionizing radiation on proteins has been carried out on solutions and little detailed information is available of the effect on insoluble fibrous proteins.

Four samples of ground ox-hide collagen, sealed in tubes in atmospheres of oxygen or nitrogen and with moisture contents of 5 and 80%, were given 5×10^7 rads of γ-irradiation.

Apart from a slight yellowing no visible change took place during irradiation in those samples containing 5% moisture. When immersed in water they swelled to a soft gel which slowly dissolved. Collagen with 80% moisture swelled during irradiation to a firm gel. This was not observed to shrink or dissolve even in boiling water.

In cold 0·1N-acetic acid about 95% of the irradiated collagen containing 5% moisture dissolved in 24 hr., compared with 5% dissolution of the irradiated samples containing 80% moisture, and 1% dissolution of the control. Dialysis indicated the presence of a large proportion of low molecular weight material in the solutions.

In all samples there was an overall 10–15% loss of amino acid residues. Losses of individual amino acids varied considerably, but the basic compounds and those with a ring structure seemed to possess a sensitivity to radiation higher than the average.

There was some loss of nitrogen but little free ammonia was found on opening the tubes. There was also an 80–100% increase in amide-N, suggesting breakdown of peptide chains, but only small amounts of keto acids were detected. Evidence of peptide-bond rupture of a different nature was obtained by a study of the *N*-terminal amino groups, which increased to 2–4 times that of the control.

This evidence is consistent with a loss of molecular structure and decrease in chain length. In the wet state, at least, formation of some new crosslinks occurs to stabilize the molecule.

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Ribonucleic Acid Synthesis in Extracts of Mammalian Cells.

By R. H. Burdon* and R. M. S. Smellie. (*Department of Biochemistry, The University, Glasgow, W. 2*)

In a previous report (Burdon & Smellie, 1960), conditions were described for the incorporation of [5:6-$^3H_2$]uridine into ribonucleic acid (RNA) by particle-free extracts of Ehrlich ascites carcinoma cells. Further studies have shown that the addition of reduced diphosphopyridine nucleotide (reduced DPN) and triphosphopyridine nucleotide (TPN) greatly stimulates this incorporation and analysis of the products of alkaline hydrolysis of the RNA has shown that this is due to an increase in the amount of [^{3}H]uridine attached to the RNA in a terminal position. Studies on the acid-soluble

fractions have demonstrated that the reduced DPN and TPN promote the formation of labelled uridine triphosphate (UTP) from [^{3}H]uridine.

The uptake of [^{3}H]uridine is also stimulated by supplementation of the reaction medium with a mixture of the diphosphates of adenosine, guanosine and cytidine (ADP, GDP and CDP) or with the triphosphates of guanosine and cytidine (GTP and CTP).

RNA prepared from the particulate fraction of the disrupted-cell preparation augments uridine incorporation, probably by acting as a primer, whereas addition of comparable amounts of RNA prepared from the cell sap has a similar but much less pronounced effect.

Dialysis of the extracts has little effect on the incorporation of [^{3}H]uridine but pre-incubation (prior incubation of the extracts alone at 37° for 30 min.) is inhibitory. The net synthesis of RNA observed with preparations of dialysed extract in the presence of Mg^{2+}, TPN, reduced DPN, ATP, UTP, GTP and CTP is negligible, but in experiments with pre-incubated extract 30% net synthesis was obtained, compared with 18% for normal extracts.

Analogous ability to incorporate [^{3}H]uridine into RNA has been demonstrated in particle-free extracts of immature rabbit tissues (thymus, spleen, brain, intestine, testes, bone marrow, appendix, kidney and muscle, in order of decreasing activity). In most of these tissues the ratio of uridine incorporated in a terminal position to that in a non-terminal position of the RNA is quite low (0·1), but in brain the value was 1·0 and in muscle 2·1, indicating a much greater proportion of terminal addition in these two tissues. In brain about 46% of the total activity of the RNA was recovered in cytidine derivatives as compared with from 10 to 20% in the other tissues.

REFERENCE

Burdon, R. H. & Smellie, R. M. S. (1960). *Biochem. J.* **76**, 2P.

The Enzymic Polymerization of D-Galactose.

By M. J. Clancy and W. J. Whelan. (*The Lister Institute of Preventive Medicine, London, S.W.* 1)

The complex nature of the products obtained by the enzymic polymerization of a monosaccharide was first shown by Peat, Whelan & Hinson (1952), who re-examined the synthesis of gentiobiose from D-glucose by almond emulsin (β-glucosidase) and isolated all five β-linked disaccharides of glucopyranose. In a later investigation, these workers (Peat, Whelan & Hinson, 1955) reported that an enzyme preparation from *Aspergillus niger*, containing α- and β-glucosidases, catalysed the synthesis from D-glucose of a mixture of α- and β-linked disaccharides, from which five α-linked and three β-linked disaccharides were isolated and identified. Shibasaki (1955) observed that when D-glucose was treated with an acetone-dried preparation of *Schizosaccharomyces pombe* no. 2, isomaltose, nigerose, and kojibiose were synthesized.

In the case of D-galactose, Bourquelot & Aubry (1916, 1917) isolated two galactobioses from the product of the action of almond emulsin on this sugar; these would appear to have been the β-1:6- and the β-1:4-linked galactobioses. The polymerization of D-galactose by yeast α-galactosidase has been reported but no experimental details were given (French, 1954).

The action of the α-galactosidase of *Saccharomyces carlsbergensis* (brewer's yeast) on a concentrated solution of D-galactose has now been examined in detail. It was found that an enzyme preparation obtained by washing the yeast cells with water until free from reducing power, which could be used directly as such or preserved until needed by drying with acetone, was a convenient source of α-galactosidase, free from β-galactosidase activity. Chromatographic analysis of digests showed that the products obtained when the yeast cells were incubated with D-galactose in the presence of toluene differed in composition from those produced when no toluene was used.

The mixture of sugars obtained when toluene was used was shown to contain galactose and di-, tri-, and higher oligo-saccharides. This mixture was fractionated by adsorption chromatography on charcoal, partition chromatography on cellulose, and in some instances final purification was effected by use of zone electrophoresis on paper. The total yield of products suggested that a considerable portion of the galactose was fermented by the yeast cells. Of the initial galactose, 7·5% was polymerized and about 60% of the oligosaccharide fraction consisted of 6-*O*-α-D-galactopyranosyl-D-galactose. In addition, galactobioses which have been isolated, identified and characterized are the α-1:5-, α-1:4- and α-1:3-linked disaccharides. Other disaccharides were isolated but not in sufficient quantity to permit characterization. Two trisaccharide fractions were isolated but each of these were mixtures and it was not found possible to resolve them.

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REFERENCES

Bourquelot, É. & Aubry, A. (1916). *C.R. Acad. Sci., Paris*, **163**, 60.
Bourquelot, É. & Aubry, A. (1917). *C.R. Acad. Sci., Paris*, **164**, 443, 521.
French, D. (1954). *Advanc. Carbohyd. Chem.* **9**, 158.
Peat, S., Whelan, W. J. & Hinson, K. A. (1952). *Nature, Lond.*, **170**, 1056.
Peat, S., Whelan, W. J. & Hinson, K. A. (1955). *Chem. & Ind.*, p. 385.
Shibasaki, K. (1955). *Tohoku J. agric. Res.* **6**, 171.

Renal-Tissue Citrate and Citrate Excretion in the Rat.

By M. A. Crawford* and M. D. Milne. (*Department of Medicine, Postgraduate Medical School, London, W.* 12)

Ostberg (1931) first showed that sodium bicarbonate could increase citrate excretion. Amongst the many other salts shown to increase citrate excretion (Orten & Smith, 1937), Krebs, Salvin & Johnson (1938) claimed that malonate, by the inhibition of succinic dehydrogenase, caused an accumulation of succinic, α-ketoglutaric and citric acids within tissue, and subsequently an increased excretion. Orten & Smith (1939) demonstrated a large increase in renal-tissue and urinary citrate after injection of sodium malate. As they found no increase in blood, liver or muscle they maintained that the kidney was the site of citrate synthesis.

Evidence has accumulated suggesting that the kidney is responsible for removing circulating citrate (Martensson, 1940; Freeman & Chang, 1950; Herndon & Freeman, 1958).

Crawford, Milne & Scribner (1959) showed that an injection of potassium bicarbonate resulted in an increase of renal-tissue and urinary but not blood citrate (cf. Orten & Smith, 1939). They also confirmed previous findings that the excretion of citrate was dependent on intracellular pH as opposed to urinary pH (Clarke, Evans, MacIntyre & Milne, 1955).

Experiments to be reported now suggest that citrate excretion is not necessarily a function of 'renal-tissue level', but that the rate of excretion of citric, α-ketoglutaric and probably all the acids of the tricarboxylic acid cycle, is dependent more on the acid–base balance of renal tissue than the nature of the substance injected.

An injection of 8 m-moles of $KHCO_3$/kg. resulted in an increase in urinary citrate from a mean of 1·6 μmoles/kg./hr. to 46·5 μmoles/kg./hr. Renal-tissue citrate rose from 0·23 μmole/g. to 1·61 μmoles/g. within 2 hr. Fluoroacetate (0·66 mg./kg.) increased kidney citrate to 1·54 μmoles/g. but, unlike malonate, did not alter urinary citrate.

The urinary citrate response to potassium bicarbonate was unaltered by the presence of fluoroacetate, i.e. 46·1 μmoles/kg./hr., despite a doubly increased kidney level of 3·05 μmoles/g. The kidney citrate after fluoroacetate was recoverable from the mitochondria but not after injection of potassium bicarbonate alone.

A 20-fold increase in urinary α-oxoglutarate occurred after injection of 6 m-moles of $KHCO_3$ or sodium malate/kg., and was unaffected by the presence of fluoroacetate. Under the conditions employed, sodium succinate, malate, malonate and carbonate produced quantitatively similar increases in citrate excretion.

REFERENCES

Clarke, E., Evans, B. M., MacIntyre, I. & Milne, M. D. (1955). *Clin. Sci.* **14**, 421.
Crawford, M. A., Milne, M. D. & Scribner, B. H. (1959). *J. Physiol.* **149**, 413.
Freeman, S. & Chang, T. S. (1950). *Amer. J. Physiol.* **160**, 335.
Herndon, R. F. & Freeman, S. (1958). *Amer. J. Physiol.* **192**, 369.
Krebs, H. A., Salvin, E. & Johnson, W. A. (1938). *Biochem. J.* **32**, 113.
Martensson, J. (1940). *Acta physiol. scand.* **1**, suppl. 2.
Orten, J. M. & Smith, A. H. (1937). *J. biol. Chem.* **117**, 555.
Orten, J. M. & Smith, A. H. (1939). *J. biol. Chem.* **128**, 101.
Ostberg, O. (1931). *Skand. Arch. Physiol.* **62**, 81.

The Presence of Basic Proteins in Ribonucleoproteins of Rat Liver.

By P. Cohn*, Pamela Simson and J. A. V. Butler. (*Chester Beatty Research Institute, Institute of Cancer Research; Royal Cancer Hospital, London, S.W.* 3)

In the course of fractionations of the proteins of rat-liver microsomes with the detergents, Lubrol W, sodium perfluoro-octanoate and sodium deoxycholate (Cohn & Butler, 1958) analyses were made of the various residual protein fractions. The total protein of the microsome was not predominantly basic, but the proportion of basic amino acids increased in the fractions not solubilized by these treatments in the order given above (Butler, Cohn & Simson, 1960). However, when the perfluorooctanoate and deoxycholate pellets were extracted with 0·2 N-HCl, basic proteins were obtained in which the molar ratios of basic:acidic amino acids were 1·3–1·5, which may be compared with the value of 2 for the total histone of calf-thymus. The principal *N*-terminal groups of the extracted proteins were alanine 23%, proline 24%, glycine 15%; cf. proline 50%, alanine 36% in a typical unfractionated histone. It therefore appears that

the residual ribonucleoprotein contains proteins approximating in composition to histones, which are present in comparatively large amounts, e.g. 20–30 % of the protein of the perfluoro-octanoate pellet.

REFERENCES

Butler, J. A. V., Cohn, P. & Simson, P. (1960). *Biochim. biophys. Acta*, **38**, 386.
Cohn, P. & Butler, J. A. V. (1958). *Biochem. J.* **70**, 254.

The Effect of Cobalt on the Metabolism of Rat-Dermal Fibroblasts in Culture.

By Mary Daniel, J. T. Dingle* and M. Webb. (*Strangeways Research Laboratory, Cambridge*)

To obtain further information on the biological action of cobalt, a metal of known carcinogenic activity *in vivo*, a study has been made of the effect of Co^{2+} on mammalian cells *in vitro*. Repeated subculture of rat-dermal fibroblasts with the intermittent addition of 5 μg. of Co^{2+}/ml. (as $CoCl_2$) has yielded after a period of 6 months cells which differ cytologically and biochemically from the controls. These cobalt-treated cells, which show some of the morphological characteristics of malignant cells, are less susceptible to inhibition of growth by Co^{2+}, and exhibit a decreased respiration and increased aerobic and anaerobic glycolysis. These changes persist on prolonged subculture of the cells in the absence of Co^{2+}.

An immediate response to Co^{2+} is observed with freshly isolated (untreated) fibroblasts. Thus, in these cells, in contrast to those of the cobalt-tolerant line, a three-fold rise in glycolysis is observed after cultivation for 48 hr. in the presence of 2·5 μg. of Co^{2+}/ml. This concentration of Co^{2+} completely inhibits respiration in the untreated cells, whilst twice the amount is needed to arrest the respiration of the cobalt-tolerant line.

Inhibition of respiration is apparent 6–8 hr. after the addition of Co^{2+} to cultures of freshly isolated fibroblasts. Though the respiratory inhibition may be complete at 8 hr., no effect on glycolysis is observed at this time.

The Distribution of Additional Components in Ribonucleic Acid from Particulate and Soluble Fractions of Cells.

By D. B. Dunn, J. D. Smith and M. V. Simpson. (*Agricultural Research Council, Virus Research Unit, Huntingdon Road, Cambridge*)

Previous studies (Dunn, 1959) have shown that a number of minor components present in ribonucleic acid (RNA) from a variety of sources are present in comparatively high proportions in the RNA from the soluble fraction of rat liver (S-RNA). We have now shown that S-RNA from *Escherichia coli* prepared by Tissières (1959) contains high proportions of several of these components (Dunn, Smith & Spahr, 1960). As in the rat-liver S-RNA, the additional component present in the highest proportion was pseudo-uridine. This represented 14 mol./100 mol. of uridylic acid. A similar proportion of this compound has also been shown to be present in S-RNA from yeast (Otaka, Hotta & Osawa, 1959). With the exception of 1-methylguanine, however, the other additional components in the S-RNA from *E. coli* were different from those found in the rat-liver material.

A number of additional components were detected in the microsomal fraction from rat liver (Dunn, 1959). Similar proportions of the same compounds appear to be present in the RNA of washed microsomal particles prepared from this fraction with 0·5 % deoxycholate by the method of Littlefield, Keller, Gross & Zamecnik (1955). We are investigating the possibility that these additional components are in a low-molecular-weight RNA in the particles. However, other studies we have made on pig-liver RNA using fractionation with sodium chloride (Laskov, Margoliash, Littauer & Eisenberg, 1959) suggest that at least pseudouridine and 2-*O*-methylribose are present in high-molecular-weight RNA. In contrast to the mammalian ribonucleoprotein particles, the 70s particles from *E. coli* (Tissières, Watson, Schlessinger & Hollingworth, 1959) were found to be almost free from additional components. The only compound detected was pseudo-uridine and this represented only 0·8 mol. per 100 mol. of uridylic acid.

As a high proportion of additional components seems to be characteristic of S-RNA we are investigating whether mitochondria which have been shown to be capable of carrying out net protein synthesis (Bates & Simpson, 1959) contain an RNA with this type of composition.

We wish to thank Dr A. Tissières for providing us with the S-RNA and 70s particles from *E. coli*.

REFERENCES

Bates, H. M. & Simpson, M. V. (1959). *Biochim. biophys. Acta*, **32**, 597.
Dunn, D. B. (1959). *Biochim. biophys. Acta*, **34**, 286.
Dunn, D. B., Smith, J. D. & Spahr, P. F. (1960). *J. mol. Biol.* (in the Press).
Laskov, R., Margoliash, E., Littauer, U. Z. & Eisenberg, H. (1959). *Biochim. biophys. Acta*, **33**, 247.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Otaka, E., Hotta, Y. & Osawa, S. (1959). *Biochim. biophys. Acta*, **35**, 266.
Tissières, A. (1959). *J. mol. Biol.* **1**, 365.
Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959). *J. mol. Biol.* **1**, 221.

Kinetics of the Trypsin-catalysed Hydrolysis of Synthetic Substrates.

By D. T. ELMORE and N. J. BAINES. (*Department of Biochemistry, Queen's University, Belfast, Northern Ireland*)

Kinetic studies of the trypsin-catalysed hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester (BAEE) and N^{α}-toluene-*p*-sulphonyl-L-arginine methyl ester (TAME) have been reported by Gutfreund (1955) and Ronwin (1959). Determination of $V_{max.}$ at different pH values suggested the participation of a group with pK'_a 6·25 in the activated complex formed during the hydrolysis of BAEE. In the hydrolysis of TAME (in absence of Ca^{2+}), however, Ronwin (1959) found that a group having pK'_a about 7·5 was involved. We have independently studied the hydrolysis of TAME under slightly different conditions and have partly confirmed Ronwin's results. Our determinations of the pK'_a at different temperatures indicate that the heat of ionization of the group is about 8 kcal./mole, but interpretation of these data require caution (cf. Bruice & Schmir, 1959). Under our conditions, K_m [i.e. $(k_2+k_3)/k_1$] for the hydrolysis of TAME is lower than that reported by Martin, Golubow & Axelrod (1959) and increases from pH 6·6 to 8·4. Hence, k_3 is comparable in magnitude with k_2, unlike the case of BAEE (Gutfreund, 1955). Determination of k_3 for TAME and N^{α}-toluene-*p*-sulphonyl-L-homoarginine methyl ester (THAME) at pH 8·40 and various temperatures has permitted the calculation of enthalpies and entropies of activation. For TAME at 25°, k_3, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and K_m are, respectively, 100 moles/sec./mole of trypsin, 8·4 kcal./mole, −16·5 e.u., $1{\cdot}2 \times 10^{-4}$ M. The corresponding values for THAME are 4·0 moles/sec./mole of trypsin, 9·2 kcal./mole, −20·2 e.u., and $2{\cdot}6 \times 10^{-4}$ M.

Cholate anion accelerates the hydrolysis of TAME and THAME by trypsin. The value of k_3 for TAME is increased to 133 moles/sec./mole of trypsin by 2 mM-cholate, and to 177 moles/sec./mole of trypsin by 6 mM-cholate at 25°. At the same time, $\Delta H^{\ddagger}$ is slightly increased and $\Delta S^{\ddagger}$ becomes less negative. Similar changes have been observed with THAME; in addition, 2 mM-cholate lowers K_m for THAME to $1{\cdot}1 \times 10^{-4}$ M.

Considerable difficulty has been experienced in these kinetic studies owing to the tendency of trypsin to be adsorbed or denatured on glass surfaces, particularly at slightly alkaline pH values. Similar behaviour has been reported for ribonuclease (Shapira, 1959) and chymotrypsin (Bixler & Niemann, 1959).

REFERENCES

Bixler, R. L. & Niemann, C. (1959). *J. Amer. chem. Soc.* **81**, 1412.
Bruice, T. C. & Schmir, G. L. (1959). *J. Amer. chem. Soc.* **81**, 4552.
Gutfreund, H. (1955). *Trans. Faraday Soc.* **51**, 441.
Martin, C. J., Golubow, J. & Axelrod, A. E. (1959). *J. biol. Chem.* **234**, 1718.
Ronwin, E. (1959). *Biochim. biophys. Acta*, **33**, 326.
Shapira, R. (1959). *Biochem. biophys. Res. Commun.* **1**, 236.

The Mechanism of Ketone-Body Formation.

By F. J. R. HIRD* and R. H. SYMONS. (*Department of Biochemistry and School of Agriculture, University of Melbourne, Victoria, Australia*)

Currently there are two mechanisms proposed for conversion of acetoacetyl-coenzyme A into acetoacetate; by direct hydrolysis (Drummond & Stern, 1960) and by its prior conversion into hydroxymethylglutaryl-coenzyme A, with cleavage of the latter compound to acetoacetate and acetyl-coenzyme A (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958).

Several features of the mechanism proposed by Lynen *et al.* (1958) allow it to be detected in whole cells. The acetyl-coenzyme A oxidized by the tricarboxylic acid cycle may originate from acetoacetyl-coenzyme A in two ways, by the thiolase reaction (producing two molecules of acetyl-coenzyme A) and by cleavage of hydroxymethylglutaryl-coenzyme A formed by condensation. The acetyl-coenzyme A produced by the cleavage reaction will be derived only from C-1 and C-2 of the original acetoacetyl-coenzyme A, C-3 and C-4 remaining to continue in their respective positions in acetoacetate. Assuming the absence of an isotope effect, a difference in amount of respiratory CO_2 coming from separately labelled acetyl moieties of butyrate will therefore reflect the operation of the scheme proposed by Lynen *et al.* (1958).

Using omasal tissue from the sheep (Hird & Symons, 1959), with [1-^{14}C]butyrate and [3-^{14}C]-butyrate the ratios of C-1 to C-3 appearing as $^{14}CO_2$ from the tricarboxylic acid cycle were from 2·2 to 4·0. With [2-^{14}C]butyrate and [4-^{14}C]-butyrate the corresponding ratios for C-2 and C-4 were from 2·2 to 2·6. In the case of rat liver and the latter compounds the ratios found were 1·6 and 1·8.

The ratio of the incorporation of ^{14}C from [1-^{14}C]-acetate (and from [2-^{14}C]lactate) into C-1 and C-3 of acetoacetate would also allow the detection of the mechanism proposed by Lynen *et al.* (1958), as in this case also the ratio should be greater than one. With [1-^{14}C]acetate, unlabelled butyrate and omasal tissue the ratio found was from 1·9 to 3·5. For liver the figures found were 2·0 and 3·3. With [2-^{14}C]lactate and omasal tissue the figures ranged

from 3·2 to 3·8, and for liver they were 2·4 and 2·4.

The results obtained indicate that the mechanism of Lynen *et al.* (1958) is operating in the omasum and liver.

Interpretation of the results obtained, however, does not exclude direct deacylation as a component of the total synthesis of acetoacetate. Indeed, direct deacylation of the acetoacetyl-coenzyme A synthesized by the reverse thiolase reaction would not influence the detection procedure used.

REFERENCES

Drummond, G. I. & Stern, J. R. (1960). *J. biol. Chem.* **235**, 318.
Hird, F. J. R. & Symons, R. H. (1959). *Biochim. biophys. Acta*, **35**, 422.
Lynen, F., Henning, U., Bublitz, C., Sörbo, B. & Kröplin-Rueff, L. (1958). *Biochem. Z.* **330**, 269.

On the Mechanism of Activation of Trypsinogen by Penicillium kinase.

By T. Hofmann. (*Department of Biochemistry, The University, Sheffield* 10)

It was shown previously that during the activation of trypsinogen by an enzyme from *Penicillium janthinellum* four peptides and one free amino acid are produced, namely Val.$(Asp)_4$.Lys, Val.$(Asp)_2$, $(Asp)_2$.Lys, Asp.Asp and lysine (Hofmann, 1960).

A semiquantitative analysis of the peptides produced during the activation shows that the hexapeptide and both tripeptides are present shortly after the start of the activation. After full activity has been reached the hexapeptide slowly disappears and gives rise to the two tripeptides. One of these, $(Asp)_2$.Lys, is further hydrolysed to give Asp.Asp and lysine.

This is compatible with the scheme of activation shown at foot of page.

It has not been possible so far to establish whether the proposed intermediate between trypsinogen and trypsin, $(Asp)_2$.Lys.Ileu.protein, has enzymic activity or not. This mechanism differs from that proposed recently (Nakanishi, 1959) for the activation of trypsinogen by an enzyme from *Aspergillus oryzae*. This enzyme liberates the hexapeptide which is then broken down into valine, the tetrapeptide $(Asp)_4$, and lysine.

The activation of trypsinogen by an enzyme from *A. saitoi* (Rovery, Gabeloteau, Guy & Desnuelle, 1958) gives rise to 0·5 mole of valine as *N*-terminal amino acid for 1 mole of isoleucine per mole of trypsinogen. The *P. janthinellum* enzyme also causes the appearance of new *N*-terminal amino acids in the protein, in addition to isoleucine. For every mole of isoleucine approximately 1 mole of valine, 0·8 mole of serine, 0·35 mole of alanine and 0·5 mole of aspartic acid were found, after complete activation.

REFERENCES

Hofmann, T. (1960). *Biochem. J.* **74**, 41P.
Nakanishi, K. (1959). *J. Biochem., Tokyo*, **46**, 1553.
Rovery, M., Gabeloteau, C., Guy, O. & Desnuelle, P. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, **8**, 79.

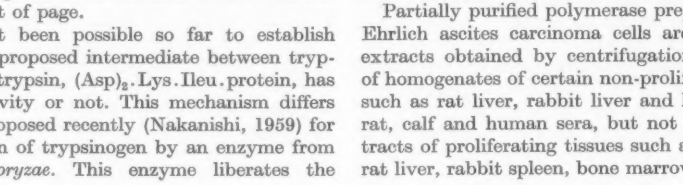
Factors Controlling the Enzymic Synthesis of Deoxyribonucleic Acid.

By S. M. Weissman, E. D. Gray, R. Y. Thomson, R. M. S. Smellie* and J. N. Davidson. (*Department of Biochemistry, University of Glasgow, Glasgow, W.* 2)

A polymerase which promotes incorporation of thymidine triphosphate into deoxyribonucleic acid in the presence of the triphosphates of deoxyadenosine, deoxyguanosine and deoxycytidine and primer deoxyribonucleic acid has been described (Smellie *et al.* 1960). Kinases which promote the phosphorylation of thymidine also occur widely (Weissman, Paul, Thomson, Smellie & Davidson, 1960).

In immature rabbit tissues the polymerase occurs in decreasing order of activity in bone marrow, thymus, spleen, liver and kidney.

Partially purified polymerase preparations from Ehrlich ascites carcinoma cells are inhibited by extracts obtained by centrifugation at 105 000 *g* of homogenates of certain non-proliferating tissues such as rat liver, rabbit liver and kidney and by rat, calf and human sera, but not by similar extracts of proliferating tissues such as regenerating rat liver, rabbit spleen, bone marrow or thymus.



Kinase preparations from Ehrlich ascites cells are inhibited by such extracts of resting rat and rabbit liver and, to a lesser extent, rabbit kidney and by calf serum.

Extracts prepared by high-speed centrifugation of homogenates of regenerating rat liver show much higher levels of polymerase and kinase activities than do similar extracts of normal liver and the addition of normal liver extracts strongly inhibits these systems in regenerating liver. No inhibitory activity has been found in dialysates or acid-soluble fractions of extracts of normal liver or in boiled liver extracts.

Enzymes responsible for the removal of inorganic phosphate from deoxyribonucleoside triphosphates, thymidine diphosphate and thymidine monophosphate are present in extracts of normal liver, and catalyse extensive breakdown of deoxyguanosine triphosphate and lesser breakdown of the triphosphates of deoxyadenosine, deoxycytidine and thymidine. They have not been found to be significantly different in activity in extracts of normal and regenerating liver. Poor correlation has been observed between the dephosphorylating and inhibitory activities of ammonium sulphate fractions and dialysed preparations of extracts of normal liver.

REFERENCES

Smellie, R. M. S., Gray, E. D., Keir, H. M., Richards, J., Bell, D. & Davidson, J. N. (1960). *Biochim. biophys. Acta*, **37**, 243.

Weissman, S. M., Paul, J., Thomson, R. Y., Smellie, R. M. S. & Davidson, J. N. (1960). *Biochem. J.* **76**, 1P.

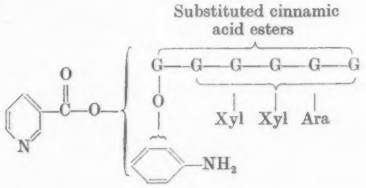
The Isolation of Niacytin, the Bound Form of Nicotinic Acid.

By E. Kodicek and P. W. Wilson. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Cereal products contain almost all their nicotinic acid in bound form unavailable to mammals or to bacteria (see Kodicek, 1951, 1960). Preparations containing 1·7–2·5% of nicotinic acid have been made from wheat and rice brans by Chaudhuri & Kodicek (1950), Chaudhuri (1954) and Guha & Das (1957).

We have now isolated from wheat bran a substance homogenous by various criteria, including Tiselius electrophoresis, countercurrent dialysis, and preparation of dinitrophenyl derivatives. Initial stages of purification (Chaudhuri & Kodicek, 1950) involving fractional precipitation with acetone, were followed by extraction with pyridine and phenol, and precipitation with acetone and 85% ethanol. The resulting brown powder (I) (30 mg. from 100 g. of wheat bran) was insoluble in many organic solvents, but soluble in water and 70% ethanol. I showed $[\alpha]_D^{17} - 15 \pm 5°$ in water (*c*, 1) (Found: C, 47·4; H, 6·1; O, 45·0; N, 1·44%; P and S absent; corr. for 2% protein impurity: C, 46·5; H, 6·0; O, 44·0; N, 1·16%); light-absorption max., 288 and 325 mμ, inflexion 262 and 274 mμ, min. 310 mμ, showing a bathocromic shift with sodium acetate typical for phenolic esters. Hydrolytic studies showed the presence of 5% nicotinic acid (NA), 48·5% glucose, 8% xylose, 4% arabinose, about 5% substituted aromatic amine (possibly *o*-aminophenol) (ArA), about 27% phenolic esters (including ferulic and sinapic acids), and not more than 2% protein, almost certainly an impurity. The analyses suggest 1 mole NA:1 mole ArA:6 moles glucose:2 moles xylose:1 mole arabinose:about 3 moles substituted cinnamic acids.

Nicotinic acid was readily liberated from I by 0·1N-alkali and slowly by liver esterase, but scarcely at all by 0·1N-HCl. Ammonolysis indicated that nicotinic acid was bound by an ester link through its carboxyl group, the ring nitrogen remaining trivalent. Partial hydrolysis with N-HCl yielded compound II (NA_1ArA_1 glucose$_4$), and compound III (NA_1ArA_1 glucose$_2$). Neither of these was available as a source of nicotinic acid for *Lactobacillus casei*. Snail cellulase (Myers & Northcote, 1959) slowly removed glucose from I, indicating the presence of β-glycosidic linkages. II could be converted by acid treatment into III. That the primary aromatic amine had an unsubstituted amino group in I, II and III was confirmed by standard reactions, including diazotization and coupling with β-naphthol, and the carbylamine test.



The empirical formula (corrected for 0·28% α-amino N impurity), $C_{94}H_{142}O_{66}N_2$, requires C, 47·9; H, 6·1; O, 44·8; N, 1·19%, giving a mol.wt. of 2370, while a 5% content of nicotinic acid indicates a mol.wt. of 2460. The nitrogen content is consistent with the presence of 2 atoms of nitrogen per mole plus the nitrogen from the trace of protein impurity. It appears that I is a reasonably well characterized compound, for which we suggest the accompanying tentative structure, and propose the name niacytin.

REFERENCES

Chaudhuri, D. K. (1954). *Sci. & Cult.* **19**, 409.
Chaudhuri, D. K. & Kodicek, E. (1950). *Nature, Lond.*, **165**, 1022.
Das, M. L. & Guha, B. C. (1957). *Nature, Lond.*, **180**, 1285.
Kodicek, E. (1951). *Rep. Progr. Chem.* **48**, 276.
Kodicek, E. (1960). *Brit. J. Nutr.* **14**, 13.
Myers, F. L. & Northcote, D. H. (1959). *Biochem. J.* **71**, 749.

Amino Acid Incorporation into Protein of Isolated Rat-Liver Ribosomes.

By A. KORNER.* (*Department of Biochemistry, University of Cambridge*)

It has been reported recently (Korner, 1959) that ribosomes (microsomal particles) can be isolated by treatment of rat-liver microsomes with deoxycholate (Palade & Siekevitz, 1956; Littlefield, Keller, Gross & Zamecnik, 1955) in a state in which they will incorporate radioactive amino acids into protein in an *in vitro* system of the type described by Zamecnik & Keller (1954). It has now been observed that the ribosomes are more stable than the microsomes from which they are derived, so that they can be washed free from the last traces of deoxycholate by re-suspension in a buffered salt medium followed by centrifuging, without loss of the ability to incorporate amino acids into protein. Since deoxycholate is a potent inhibitor of amino acid incorporation in this system, removal of the last traces of it enables one to obtain a preparation of ribosomes which actively incorporate amino acids into protein for some hours.

Thus, washed ribosomes suspended in 0·03 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) – hydrochloride buffer at pH 7·5 containing 0·005 M-$MgCl_2$, 0·05 M-NaCl, 0·08 M-KCl, 0·001 M-adenosine triphosphate (ATP), 0·02 M-creatine phosphate, 0·0006 M-guanosine triphosphate, 0·03 mg. of creatine phosphokinase in a final volume of 1 ml., incorporate DL-[1-^{14}C]leucine into protein for at least 3 hr. when incubated at 37° in the presence of the soluble fraction of the cell or a pH 5 enzyme preparation.

More amino acid is incorporated into the protein of ribosomes than into the protein of microsomes containing the same amount of ribonucleic acid. During incubation radioactively labelled protein is transferred from the ribosomes to the supernatant liquid.

The incorporation is, to a large extent, dependent upon the addition of the soluble fraction of the cell or a pH 5 enzyme preparation to the incubation mixture. If the ATP-generating system is omitted, full incorporation can still be obtained by increasing the ATP concentration, but concentrations of ATP (containing equimolar $MgCl_2$) above 0·005 M appear to be inhibitory.

Washed ribosomes can be dialysed, or stored at 0° for several days, without substantial loss of incorporating ability.

A good direct correlation has been noted between the percentage of the ribosomes, in media of different Mg^{2+} concentrations, which sediment in the analytical ultracentrifuge at about 78 S, and the extent of amino acid incorporation into ribosome protein.

REFERENCES

Korner, A. (1959). *Biochim. biophys. Acta*, **35**, 554.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Palade, G. E. & Siekevitz, P. (1956). *J. biophys. biochem. Cytol.* **2**, 171.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

The Metabolism of *N*-Acetyl-5-[β-^{14}C]Methoxytryptamine.

By S. KVEDER, W. M. MCISAAC and IRVINE H. PAGE. (*The Research Division, Cleveland Clinic Foundation, and The Frank E. Bunts Educational Institute, Cleveland, Ohio, U.S.A.*)

The potent frog-skin-lightening agent 'melatonin' isolated from pineal tissue has been claimed to be *N*-acetyl-5-methoxytryptamine (Lerner, Case & Heinzelman, 1959). This compound would appear to be the product of a methylating enzyme system in the pineal gland acting on the substrate *N*-acetylserotonin (Weissbach & Axelrod, 1960), which itself is a metabolite of serotonin (McIsaac & Page, 1959).

In order to study its metabolic fate, we synthesized *N*-acetyl-5-[β-^{14}C]methoxytryptamine, m.p. 117°, with a specific activity of 774·7 μc/g., identical with an authentic non-radioactive sample, mixed m.p. 117° (Found: C, 66·88; H, 6·87; N, 11·57. Calc. for $C_{13}H_{16}N_2O_2$: C, 67·22; H, 6·94; N, 12·06 %).

Albino and coloured rats were dosed with 5 mg. of *N*-acetyl-5-[β-^{14}C]methoxytryptamine orally and intraperitoneally. Neither the type of animal nor the route of administration appeared to affect the rate or pattern of metabolism. The compound was metabolized rapidly, 70 % of administered radioactivity being excreted in the urine and 20 % in the faeces in 24 hr.

Paper chromatography of the urine revealed at least two metabolites. The major metabolite (R_F values 0·40 and 0·22 in butanol–acetic acid–water, 4:1:5, by vol., and propanol–conc. ammonia, 9:1, v/v respectively) gave positive reactions with

p-dimethylaminobenzaldehyde and Brentamine Fast Red B Salt, but negative reaction with 2:6-dichloroquinonechloroimide. The chromatographic pattern was not significantly changed after incubation with β-glucuronidase. These findings indicate that hydroxylation of the indole nucleus might have occurred. The minor metabolite (R_F values 0·80 and 0·68) did not give a positive reaction with *p*-dimethylaminobenzaldehyde.

As no 5-methoxy-indole-3-acetic acid was found it would appear that de-acetylation of *N*-acetyl-5-methoxytryptamine does not occur.

REFERENCES

Lerner, A. B., Case, J. D. & Heinzelman, R. V. (1959). *J. Amer. chem. Soc.* **81**, 6084.
McIsaac, W. M. & Page, I. H. (1959). *J. biol. Chem.* **234**, 858.
Weissbach, H. & Axelrod, J. (1960). *Fed. Proc.* **19**, 50.

The Fluorimetric Assay of *N*-Acetyl-β-Glucosaminidase.

By D. H. Leaback and P. G. Walker. (*Department of Biochemistry, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Brockley Hill, Stanmore, Middlesex*)

Highly sensitive methods for the assay of β-glucuronidase (Mead, Smith & Williams, 1955), and β-glucosidase (Robinson, 1956), have been described, which employ methylumbelliferyl glycosides as substrates, and which depend upon the fluorimetric estimation of methylumbelliferone liberated by enzyme hydrolysis. These methods were considered by Levvy, Hay & Marsh (1957) to be too exacting for general use on the grounds of an apparent instability of methylumbelliferone.

The fluorimetric technique has been re-evaluated in an investigation of the hydrolysis of methylumbelliferyl *N*-acetyl-β-D-glucosaminide (prepared from acetochloroglucosamine, cf. Leaback & Walker, 1957) by testicular *N*-acetyl-β-glucosaminidase. Excitation and fluorescence spectra of methylumbelliferone ($\lambda_{max.}$ 364 and 448 mμ, respectively) and of the glycoside ($\lambda_{max.}$ 325 and 378 mμ, respectively) in 0·15M-glycine, pH 10·3, were measured with an Aminco spectrofluorimeter. On the basis of these results, filters suitable for the estimation of methylumbelliferone in the Locarte fluorimeter (Laurence, 1957) were selected, which effectively reduced the blanks due to scatter, and to the fluorescence of the glycoside.

At pH 10·3 methylumbelliferyl *N*-acetyl-β-D-glucosaminide ($\lambda_{max.}$ 317 mμ, ϵ 13 620) did not interfere with the spectrophotometric estimation of methylumbelliferone ($\lambda_{max.}$ 360 mμ, ϵ 16 720). By a combination of spectrophotometric and fluorimetric methods, but with the same volumetric technique, methylumbelliferone could be estimated over a range 0·0025–11·0 μg./ml. Methylumbelliferone was found to be stable at both pH 10·3 and under conditions of enzyme assay; a slow non-enzymic hydrolysis of the glycoside occurred under the latter conditions.

As a consequence of the ease with which methylumbelliferone could be estimated over a wide range of concentration, methylumbelliferyl *N*-acetyl-β-D-glucosaminide was considered to have advantages as a substrate over the corresponding *p*-nitrophenyl glycoside (cf. Borooah, Leaback & Walker, 1957). The hydrolysis of methylumbelliferyl *N*-acetyl-β-D-glucosaminide by a partially purified preparation of *N*-acetyl-β-glucosaminidase from ram testis was studied over a 256-fold range of substrate concentration and over a 1000-fold range of enzyme concentration. The pH optimum of the enzyme was dependent upon substrate concentration and the Michaelis constant for the substrate depended upon the pH at which it was measured. In view of the non-ionic nature of the substrate, these effects indicate the ionization of an important group (or groups) at the active site of the enzyme.

The authors are indebted to Professor R. T. Williams, Dr D. Robinson and Dr J. N. Smith for advice and use of the spectrofluorimeter.

REFERENCES

Borooah, J. K., Leaback, D. H. & Walker, P. G. (1957). *Biochem. J.* **65**, 15P.
Laurence, D. J. R. (1957). *Biochem. J.* **65**, 27P.
Leaback, D. H. & Walker, P. G. (1957). *J. chem. Soc.* p. 4754.
Levvy, G. A., Hay, A. J. & Marsh, C. A. (1957). *Biochem. J.* **65**, 203.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **61**, 569.
Robinson, D. (1956). *Biochem. J.* **63**, 39.

A Thermodynamic Approach to the Selective Effect of pH in Competitive Biosynthesis of Proteins.

By S. Lewin. (*Physical-Biochemistry Research School, South-West Essex Technical College, London, E.* 17)

Experimentally, protein biosynthesis is affected, *inter alia*, by the pH value of the medium. It is possible to give a guide, based on thermodynamic considerations, as to the effect of pH change on the probability of selective production of different proteins in a biosynthetic medium.

The probability of any biochemical process taking place depends primarily on the magnitude of the free energy change accompanying the process.

In coupled systems involving reactions which are accompanied by a free energy increase, such as those encountered in the biosynthesis of proteins, the most likely reactions to take place are those which require the smallest free-energy increase (G_t).

The free energy requirement involved in the synthesis of given protein, having an isoelectric point, *pI*, in a specified medium having a particular pH value is

$$G_t = \{G_{iso} + G_{ion}\},$$

where G_{iso} is the free energy of formation of the protein at its isoelectric point, and G_{ion} is the free energy of formation of the ionised state from the isoelectric point state to that of the specified medium.

When the synthesis of two proteins, *A* and *B*, is involved in a specified medium and pH where $(G_t)_A = (G_t)_B$, the respective probabilities of formation are equal even though their respective *pI* values may not be equal. When, however, $(G_t)_A \neq (G_t)_B$ then the lower the G_t value, the greater is the probability of formation.

A particular case arises when two proteins having different isoelectric points, but having equal values of (G_{iso}) and similar titration curves, are biosynthesized in a medium of pH value which is intermediate between the two isoelectric point values. In this case the two proteins should be formed in equal proportions. A change in the pH of the medium, other things being equal, towards the value of the isoelectric point of one protein, by decreasing the charge on *A*, while increasing that of *B*, results in increased probability of synthesis of *A* and decreased probability of synthesis of *B*.

Parallel variations and applications in other fields, including genetic controls and evolution will be discussed elsewhere. The application of this theorem to the results involved in the effect of pH on the sex ratio has already been considered (Lewin, 1960).

REFERENCE

Lewin, S. (1960). *Mem. Soc. Endocrinology*, no. 7, p. 100.

The Metabolism of 5-[β-^{14}C]Methoxytryptamine.

By W. M. McIsaac, S. Kveder and Irvine H. Page. (*The Research Division, Cleveland Clinic Foundation, and The Frank E. Bunts Educational Institute, Cleveland, Ohio, U.S.A.*)

The isolation of 5-methoxyindole-3-acetic acid from pineal tissue (Lerner *et al.* 1959) raises the interesting problem of the biogenesis of this compound. The recent demonstration of an enzyme system in the pineal gland which is capable of *O*-methylating serotonin (Weissbach & Axelrod, 1960) suggests that 5-methoxytryptamine might well be the precursor of 5-methoxyindole-3-acetic acid as previously indicated by Erspamer (1955). This has been confirmed in the following way.

5-[β-^{14}C]Methoxytryptamine, m.p. 120°, with a specific activity of 988·46 μc/g. was synthesized. This compound administered to rats intraperitoneally in doses of 3 mg. was found to be rapidly metabolized, 80 % of the activity being excreted in the urine within 24 hr.

Tissue distribution studies in animals indicated that the compound was rapidly circulated, 16·3 % of the activity being found in the blood within 30 min. of administration and the level remaining above 6 % for 6 hr. Liver and kidney were the most active tissues and the brain contained 0·6 % of the dose within 30 min.

Paper chromatography of the urine revealed one major radioactive metabolite, 5-methoxyindole-3-acetic acid (R_F 0·18 and 0·88 in propanol–conc. ammonia, 9:1, v/v and butanol–acetic acid–water, 4:1:5, by vol., respectively) and a small amount of unchanged 5-methoxytryptamine (R_F 0·60 and 0·66).

Isotope-dilution studies showed that 5-methoxyindole-3-acetic acid represented 96·8 ± 1·8 % of the metabolic fate of 5-methoxytryptamine.

REFERENCES

Erspamer, V. (1955). *J. Physiol.*, **127**, 118.
Lerner, A. B., Case, J. D., Biemann, K., Heinzelman, R. V., Szmuszkovicz, J., Anthony, W. C. & Krivas, A. (1959). *J. Amer. chem. Soc.* **81**, 5264.
Weissbach, M. & Axelrod, J. (1960). *Fed. Proc.* **19**, 50.

The Effect of Encephalomyocarditis Virus Infection on the Ribonucleic Acid Metabolism of Krebs II Mouse Ascites Cells.

By E. M. Martin. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Sanders, Huppert & Hoskins (1958) have described how mouse encephalomyocarditis virus (EMC) may be readily propagated *in vitro* using suspensions of Krebs II mouse ascites cells. When EMC, a small ribonucleic acid (RNA) virus, infects this host cell it causes a marked inhibition of its RNA-turnover rate, as measured by incorporation of [6-^{14}C]orotic acid, particularly in the period just before the appearance of intracellular virus. This is not accompanied by any changes in the net amounts of RNA, DNA or protein per cell. An explanation of this inhibition was sought by examining the effects of infection on the RNA metabolism of the individual sub-cellular components.

When infected ascites cells are disrupted by osmotic shock most of the intracellular virus, which begins to appear 4 hr. after infection, sediments with the mitochondrial fraction. As the amount of virus in this fraction increases with time, so the rate of incorporation of [6-^{14}C]orotic acid into its RNA increases, reaching 320% of the uninfected control at 6 hr. The pattern of incorporation into microsomal RNA is similar to that of mitochondria, although less pronounced, as it contains less virus. Cell-sap RNA turnover is initially inhibited, but shows some stimulation in the later stages of infection.

The major site of RNA synthesis in the ascites cell is the nucleus, with a turnover rate of 100 times that of the microsomes. Nuclear RNA turnover is progressively inhibited by EMC infection, falling to only 10% of the control 6 hr. after infection. This inhibition appears most pronounced in that fraction of nuclear RNA which is soluble in 0·1M-phosphate buffer, pH 7·1, and this RNA fraction has the highest turnover rate within the cell. Infection causes an increase in size and decrease in specific activity of the intranuclear nucleotide pool. There is also a progressive decrease in the amount of nuclear buffer-soluble RNA (40% of control at 3·5 hr. after infection), while the amount of buffer-insoluble nuclear RNA is little affected. This loss of RNA is compensated by a rise in mitochondrial RNA of a similar magnitude.

The significance of this involvement of the nucleus in EMC virus replication was discussed.

REFERENCE

Sanders, F. K., Huppert, J. & Hoskins, J. M. (1958). *Symp. Soc. exp. Biol.* **12**, 123.

The Pathway of Formation of Uridine Diphosphogalacturonic Acid in Type XXXIII Pneumococci and its Importance in the Formation of Binary Capsulated Type III–XXXIII Pneumococci During Transformation.

By G. T. Mills, E. E. B. Smith, H. P. Bernheimer, R. Austrian and B. Galloway. (*Department of Medicine, State University of New York, College of Medicine at New York City, Brooklyn* 3, *N.Y., U.S.A.*)

It was shown by Austrian & Bernheimer (1959) that, on transformation of certain strains of non-capsulated Type III pneumococci with deoxyribonucleic acid (DNA) from capsulated Type I pneumococci, a small proportion of binary capsulated Type I–III cells were recovered, in addition to the expected Type I transformants. The formation of the binary capsulated Type I–III cells was explained (Austrian, Bernheimer, Smith & Mills, 1959; Mills, 1960) by the simultaneous presence in the same cell of the Type I capsular genome and the mutated Type III capsular genome. The formation of uridine diphosphoglucuronic acid (UDPGA) by the enzyme systems controlled by the former allows a bypass of the block in the mutated Type III pathway, which affects the activity of uridine diphosphoglucose (UDPG) dehydrogenase.

It was also observed that an analogous phenomenon occurs when certain non-capsulated Type III pneumococci are transformed with DNA from a capsulated Type XXXIII pneumococcus. In this case binary capsulated Type III–XXXIII cells are recovered in addition to the expected Type XXXIII. The suggestion was made that, in the formation of the binary cell, each parental type must include a uronic acid in its capsular polysaccharide and that the pathway of uronic acid formation was of importance to the phenomenon.

An examination of the capsulated Type XXXIII pneumococcus was undertaken to ascertain whether any of the biochemical pathways in this organism were similar to those in Type I pneumococci.

Chromatographic analysis of Type XXXIII capsular polysaccharide shows the presence of glucose, galactose and galacturonic acid. In addition to these findings, Type XXXIII pneumococci possess uridyl transferase, uridine diphosphogalactose-4-epimerase, UDPG dehydrogenase and UDPGA-4-epimerase, enzymes that indicate a pathway of galacturonic acid formation similar to that in Type I.

The UDPG dehydrogenase was found to have optimum activity at pH 8·4, to be activated by Mg^{2+}, and to be similar in other respects to the enzyme of Type I pneumococci (Smith, Mills, Austrian & Bernheimer, 1960).

It appears likely, therefore, that the formation of binary capsulated Type III–XXXIII pneumococci is explicable by a mechanism similar to that proposed for the case of Type I–III cells.

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REFERENCES

Austrian, R. & Bernheimer, H. P. (1959). *J. exp. Med.* **110**, 571.

Austrian, R., Bernheimer, H. P., Smith, E. E. B. & Mills, G. T. (1959). *J. exp. Med.* **110**, 585.

Mills, G. T. (1960). *Fed. Proc.* **19** (in the Press).

Smith, E. E. B., Mills, G. T., Austrian, R. & Bernheimer, H. P. (1960). *J. gen. Microbiol.* **22**, 265.

Kinetics of Phosphatase Action on Naphthyl Phosphates, Determined by a Highly Sensitive Spectrofluorimetric Technique.

By D. W. Moss.*† (*Department of Chemical Pathology, Postgraduate Medical School, London, W. 12*)

In alkaline solution, the fluorescence excitation and emission spectra of α- and β-naphthols and of their respective orthophosphate esters differ sufficiently to permit the spectrofluorimetric determination of small amounts of the free naphthols in the presence of large excesses of the esters. For the determination of α-naphthol in a solution containing α-naphthyl phosphate an activation wavelength of 335 mμ is chosen, with fluorescence measurement at 455 mμ (α-naphthyl phosphate is excited at 295 mμ and has peak emission at 365 mμ). The corresponding wavelengths for measurement of β-naphthol in the presence of its phosphate are 350 and 425 mμ, respectively (β-naphthyl phosphate has activation peaks at 280 and 320 mμ and a fluorescence peak at 340 mμ). The high sensitivity of the method enables short reaction times to be used in kinetic studies of the action of phosphatases on the naphthyl phosphates, giving a close approximation to the initial velocity. In determining the apparent K_m values of the alkaline phosphatases of serum, the velocity at the optimum pH for each substrate concentration has been used (Motzok, 1959) in the presence of 5 mM-Mg^{2+}. Apparent K_m values of 0·5 mM (α-naphthyl phosphate) and 0·4 mM (β-naphthyl phosphate) have been found for the alkaline phosphatase of serum from cases of Paget's disease, the β-isomer being hydrolysed 1·2 times as fast as the α-isomer at maximum velocity. At 5 mM substrate concentration the pH optima are 10·0 for hydrolysis of α-naphthyl phosphate and 10·1 for β-naphthyl phosphate. Somewhat different values have been found for sera from conditions with liver involvement.

Spectrofluorimetric methods of measuring acid and alkaline phosphatase activity of serum using α-naphthyl phosphate as substrate have been developed, and show greatly increased sensitivity when compared with colorimetric procedures. With an incubation time of 15 min. at 37° the alkaline phosphatase activity of as little as 0·01 ml. of normal serum can be estimated, while with a similar hydrolysis period 0·1 ml. of serum is sufficient for the determination of normal acid phosphatase activity.

The author thanks Professor E. J. King for his advice and encouragement, and Miss P. R. N. Kind for preparing the naphthyl phosphates.

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REFERENCE

Motzok, I. (1959). *Biochem. J.* **72**, 169.

Fluorine Compounds in African Plants.

By Rudolph A. Peters.* (*Department of Biochemistry, University of Cambridge and the Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham*)

The discovery of fluoroacetic acid in the leaves of the South African plant, *Dichapetalum cymosum* (Gifblaar) by Marais (1944) and of fluoro-oleic acid as well as traces of another long chain fluoro fatty acid in the seeds of the West African plant, *D. toxicarium* (ratsbane) (Peters, Hall, Ward & Sheppard, 1960), has raised biochemical problems of botanical interest. Among these is the question whether the presence of long chain fluoro fatty acids in the seeds is peculiar to *D. toxicarium* or whether it is due to storage of fluoroacetic acid in this form in the seeds. We have examined a few seeds of *D. cymosum* for which the author is much indebted to Dr Dyer; no fluoro-oleic acid was present, and virtually no fluorine (Peters & Hall, unpublished results). Hence, the presence of fluoro-oleic acid in the seeds of *D. toxicarium* is not a general phenomenon, and appears to be genetically determined. A preliminary survey of some other African plants of the same genus has been made for aliphatic carbon–fluorine compounds. These have been *D. ruhlandii*, *D. mossambicense* and *D. stuhlmanii* from East Africa, kindly sent by Dr Greenway; and *D. thonneri De Wild* (two specimens) and *D. flabellatiflorum Haumann* from the Belgian Congo, obtained through the kind help of Professor Z. M. Bacq and Dr Jurion. So far none has shown the toxicity of *D. cymosum* or *D. toxicarium*, although slight effects were observed with the leaves of *D. stuhlmanii* and *D. mossambicense*; the absence of toxicity could be due to the time of collection, as it is known that the leaves of *D. cymosum* vary in content of fluoroacetate at different seasons of the year, and the fluorine content of specimens in our hands has varied as much as 4 times, from 60 to 235 μg. of F/g.

Thanks are due to the Tropical Products Institute, London, for their co-operation.

REFERENCES

Marais, J. S. C. (1944). *Onderstepoort J. vet. Sci.* **20**, 67.
Peters, R. A., Hall, R. J., Ward, P. F. V. & Sheppard, N. (1960). *Biochem. J.* (in the Press).

Decomposition of Benzoate by a Photosynthetic Bacterium.

By M. H. Proctor*† and S. Scher. (*Hopkins Marine Station, Pacific Grove, California, U.S.A., and Department of Biochemistry, University of Cambridge, and Kaiser Foundation Research Institute, Richmond, California, U.S.A.*)

Recent investigations with photosynthetic bacteria have revealed several organisms able to use aromatic compounds as 'accessory hydrogen donors' (Scher & Proctor, 1960). The metabolism of benzoate by a representative *Rhodopseudomonas* sp. has been studied with resting-cell suspensions and with extracts prepared in the bacterial press of Hughes (1951).

Cells grown anaerobically on a mineral salts–benzoate medium in the light were shown, by standard manometric techniques, to oxidize immediately, in the dark, and at maximal rate, benzoate, protocatechuate, catechol, and α-oxoglutarate; none of the monohydroxybenzoic acids were attacked, neither were the 2:3- or 2:5-dihydroxybenzoic acids. Cells grown on a mineral salts–lactate medium in the light oxidized these substrates after lag periods of up to 3 hr. Extracts from cells grown anaerobically on mineral salts–benzoate medium in the light were unable to oxidize these substrates in the dark or light, but were able to catalyse the anaerobic decarboxylation of protocatechuate to catechol in the dark.

Assayed by the sensitive spectrophotometric method of Sistrom & Stanier (1954) the cell-free extracts lacked both the lactonizing and delactonizing enzymes for γ-carboxymethyl-Δ^{α}-butenolide. Stability of the ultraviolet spectra indicated that the cell extracts were inert towards protocatechuate and catechol with oxygen as electron acceptor; however, with peroxide as acceptor, spectral changes occurred which indicated opening of the aromatic ring.

In the presence of peroxide or oxygen, whole cells produced a β-oxoacid; however, cell extracts produced an α-oxoacid from catechol.

From these data the path of benzoate degradation by this organism differs from pathways described to date and is consistent with the route proceeding as follows: benzoate → protocatechuate → catechol → α-oxoacid, similar to that recently proposed by Dagley & Stopher (1959) for degradation of catechol by an aerobic Gram-negative organism.

We wish to thank Dr C. P. van Niel and Dr R. Y. Stanier for advice and experimental materials.

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REFERENCES

Dagley, S. & Stopher, D. A. (1959). *Biochem. J.* **73**, 16P.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Scher, S. & Proctor, M. H. (1960). In *Comparative Biochemistry of Photoreactive Pigments*. New York: Academic Press Inc. (In the Press.)
Sistrom, W. R. & Stanier, R. Y. (1954). *J. biol. Chem.* **210**, 821.

Metal–Peptide Complexes and Proteolytic Enzymes.

By B. R. Rabin.* (*Department of Biochemistry, University College London, W.C.* 1)

Recent investigations have demonstrated that for most metal ions of biological interest, the sites of co-ordination are identical in the complexes formed in aqueous solution by amides, dipeptides and tripeptides of simple α-amino acids without reactive side chains. The sites of co-ordination in the complexes of these ligands with Mn (II), Co (II), Zn (II) and Cd (II) are the α-amino group and the oxygen atom of the adjacent peptide (or amide) bond. Ionization of peptide hydrogen atoms can occur from the peptide and amide complexes of Cu (II), and the structure of these complexes varies with pH. The sites of co-ordination in the Cu (II) complexes from which none of the peptide hydrogen atoms has ionized are also the α-amino group and the oxygen atom of the adjacent peptide bond. This is true for complexes of Ni (II), some of which resemble the corresponding complexes of Cu (II) in their acid/base behaviour. Simultaneously with ionization of the peptide hydrogen atom from the Cu (II) complexes, the site of co-ordination at the peptide bond changes from oxygen to nitrogen. Complexes in which two or more peptide nitrogen atoms are co-ordinated to Cu (II) are purple in colour and this is the basis of the well-known biuret reaction of peptides and proteins. Unlike amino acid amides, esters of dipeptides and tripeptides, the biuret reaction has never been observed for dipeptides.

Colour changes analogous to the biuret reaction have been shown for the Ni (II) complexes of tripeptides; these can be yellow in colour under certain conditions and, as with Cu (II), the colour change is probably associated with the simultaneous co-ordination of two or more peptide nitrogen atoms. The factors governing the formation and structure of the metal complexes of amino acid amides and peptides were considered, together with some speculation on the reasons for the similarities and differences outlined above. The mechanism of action of metal-activated peptidases were also discussed.

Biochemical Studies of Thyroid Auto-Antigens.

By I. M. Roitt,* Deborah Doniach and K. Couchman. (*Courtauld Institute of Biochemistry and Institute of Clinical Research, Middlesex Hospital, London, W.* 1)

High levels of auto-antibodies directed against thyroid constituents are commonly found in the serum of patients with Hashimoto's thyroiditis and 'primary' myxoedema. The production of auto-antibodies and comparable lesions in the thyroid of animals injected with homologous thyroid extracts supports the view that the human disease is aetiologically related to the auto-immunization process (for review see Roitt & Doniach, 1960). It was thus of interest to study the nature of the thyroid auto-antigens evoking the immune response in the patients.

The precipitating antibodies are directed against thyroglobulin, and gel-diffusion studies reveal an immunological heterogeneity in this iodine-containing protein. Fluorescent-antibody staining of fixed thyroid sections by Hashimoto sera lacking anti-thyroglobulin antibodies, has revealed specific colloid fluorescence distinct in pattern from that obtained with anti-thyroglobulin-containing sera, suggesting the presence of a further auto-antigen (Balfour, Doniach & Roitt, unpublished results).

Using unfixed thyroid sections, fluorescent staining of the cytoplasm can be obtained with Hashimoto sera which react with thyrotoxic thyroid extracts in the complement-fixation test. The cytoplasmic antigen has been localized to the microsome fraction of thyroid homogenates. Treatment of this fraction with ribonuclease, phospholipase C or pyrophosphate does not affect the level of antigen which can be recovered in the pellet, but antigenic potency is completely lost after treatment with ethanol, acetone, deoxycholate or non-ionic detergents; prolonged incubation with trypsin or heating to 100° also destroys the antigen. Possible relationships between the antigen level and the gross biochemical composition of the microsome pellets have been explored.

The microsomal antigen is far more abundant in thyrotoxic than in normal glands. Quantitative complement-fixation studies combined with determinations of deoxyribonucleic acid have shown that this difference cannot be accounted for merely by the increased cellularity of the thyrotoxic gland but may be related to thyroid activity. This view is consistent with the finding of a high antigenic potency in single hyperfunctioning nodules as compared with the remainder of the gland where iodine uptake is low owing to suppression of thyroid-stimulating hormone caused by the unrestrained synthesis of thyroid hormone in the adenoma.

Experiments with monkeys to test the effect of variation in thyroid function on the concentration of the microsomal antigen in the gland were described.

REFERENCE

Roitt, I. M. & Doniach, D. (1960). *Brit. med. Bull.* no. 2: The Thyroid.

Gastric Enzymes.

By A. P. Ryle.* (*Department of Biochemistry, University of Edinburgh, Edinburgh* 8)

It is now generally recognized that pepsin is a somewhat unspecific enzyme and that pepsin preparations may contain more than one active enzyme. Two of the minor components are the parapepsins I and II (Ryle & Porter, 1959) which have low pH optima but rather narrower specificities than pepsin when acting on the *B* chain of oxidized insulin. Dr J. I. Harris (personal communication) has obtained a similar result in studies on the digestion of corticotrophin.

The specificity of pepsin and parapepsin II when acting on haemoglobin has been studied by identification of the new free amino and carboxyl groups produced. With parapepsin II, leucine (or isoleucine) is the major *N*-terminal residue produced (4 to 5 residues per mole of haemoglobin) while pepsin liberates about two residues of leucine (or isoleucine) and one residue each of alanine, serine, threonine and glutamic acid. The *C*-terminal amino acids liberated by both enzymes are mainly leucine (or isoleucine) and phenylalanine. These results support the earlier conclusion that parapepsin II has a narrower specificity than pepsin and suggest that both enzymes may show some degree of specificity towards the amino acid residue providing the carboxyl portion of the peptide bond.

The three enzymes have been allowed to act on benzyloxycarbonylalanyl-leucine and benzyloxycarbonylphenylalanylphenylalanine under the same conditions as those used by Katchalski and his colleagues (Neumann, Levin, Berger & Katchalski, 1959) in studying transpeptidations of the aminotransfer type catalysed by pepsin. The alanylleucine derivative was not hydrolysed by parapepsin I but was slowly (24 hr.) digested by pepsin and by parapepsin II (more rapidly at pH 4·0 than at pH 1·7), with the formation of free leucine and a second ninhydrin-positive component in the parapepsin digest. This material moved faster than leucine on paper chromatography in butanol–acetic acid.

Benzyloxycarbonylphenylalanylphenylalanine was slowly hydrolysed by all three enzymes, most slowly by parapepsin II, with the liberation of phenylalanine. In each case the digestion appeared to be rather faster at pH 4·0 than at pH 1·7. In the digests made with parapepsin II and with pepsin, an additional ninhydrin-positive material moving faster than phenylalanine in butanol–acetic acid was probably leucine, produced by autolysis.

Attempts to detect transpeptidation with parapepsin I and pepsin acting on acetylphenylalanyldiiodotyrosine, which they hydrolyse readily, have been unsuccessful.

The significance of these findings was discussed.

REFERENCES

Neumann, H., Levin, Y., Berger, A. & Katchalski, E. (1959). *Biochem. J.* **73**, 33.
Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.

The Purification and Composition of Type I Pneumococcal Capsular Polysaccharide.

By Evelyn E. B. Smith, B. Galloway and G. T. Mills. (*Department of Medicine, State University of New York, College of Medicine at New York City, Brooklyn* 3, *N.Y., U.S.A.*)

A method of purification of Type I pneumococcal capsular polysaccharide has been devised, resulting in a product varying in purity from 92 to 98% as evaluated by protein and nucleic acid contamination. This study was undertaken because of the genetic role which Type I pneumococcus may play in the phenomenon of binary capsulation (Austrian, Bernheimer, Smith & Mills, 1959) and the current lack of knowledge of the constituent sugars of this capsular polysaccharide.

Purification involves peptic and tryptic digestion of Type I cells, followed by precipitation of nucleic acid and capsular polysaccharide with 1 vol. of ethanol. Nucleic acid is removed from this precipitate by the method employed by Mills, Ondarza & Smith (1954) and the purified polysaccharide remaining in solution is precipitated by the addition of 2 vol. of ethanol.

Hydrolysis of the polysaccharide with N-H_2SO_4 for varying periods of time shows the liberation, within 15 min., of two components running chromatographically in a variety of solvents in the positions of galactose and fucose. A component corresponding to glucosamine is evident after hydrolysis for 1 hr., while galacturonic acid is liberated after 6–8 hr. of hydrolysis. After 8 hr. there is a rapid destruction of the identifiable sugars.

Application of the primary cysteine–H_2SO_4 reaction of Dische (1955) to the 1 hr. hydrolysate shows evidence of a methylpentose. The secondary cysteine–H_2SO_4 reaction on similarly treated material indicates the presence of a hexose behaving as galactose. Estimation of uronic acid by the carbazole reaction (Dische, 1955) shows the polysaccharide to contain 60% of uronic acid which appears to be entirely galacturonic acid by chromatographic criteria. The amino sugar content determined by the method of Rondle & Morgan (1955) is 8%, a value which is obtained maximally after hydrolysis for 90 min. with 3N-HCl. After this period, destruction of amino sugar occurs. The absorption maximum in the Rondle & Morgan reaction is that shown by hexosamines.

The total nitrogen value is 4%. This value cannot be accounted for on the basis of hexosamine, nucleic acid and protein present and it is apparent that other nitrogenous material is a constituent of the polysaccharide.

This investigation was supported by a grant [E-1018(C4)] from the National Institutes for Allergy and Infectious Diseases, United States Public Health Service.

REFERENCES

Austrian, R., Bernheimer, H. P., Smith, E. E. B. & Mills, G. T. (1959). *J. exp. Med.* **110**, 585.
Dische, Z. (1955). *Meth. of Biochem. Anal.*, **2**, 313.
Mills, G. T., Ondarza, R. & Smith, E. E. B. (1954). *Biochim. biophys. Acta*, **14**, 159.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.

The Interaction of Thyroid Hormones with Intra- and Extra-Cellular Proteins.

By J. R. Tata.*† (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The problem is reviewed under the four following headings:

(1) The interaction of thyroxine and 3:5:3′-triiodothyronine with serum proteins. Although the major serum thyroxine-binding protein (TBP) has not yet been isolated, recent findings regarding its nature and properties emphasize the following physiological significance. (*a*) Only free or non-protein-linked thyroid hormones can pass from blood to tissues. (*b*) Measurement of the level of circulating TBP can explain discrepancies observed in certain changes in the concentration of circulating thyroid hormones. (*c*) The difference in biological potencies of thyroxine and tri-iodothyronine is governed by the relative affinities with which they are bound to serum TBP.

(2) Interaction with tissue proteins without enzymic action on thyroid hormones. A group of

† Beit Memorial Fellow.

tissue proteins in skeletal muscle which bind thyroxine and tri-iodothyronine has been characterized. Relatively little is known about the nature and properties of intracellular TBP's except that they bind the hormones considerably less firmly than does serum TBP.

(3) Enzymes in the cell for which the thyroid hormones are substrates. Although a variety of pathways of metabolism of these hormones are now known, only the de-iodinating enzyme (thyroxine dehalogenase) has been partially purified, and its properties studied. Thyroxine dehalogenase prepared from rabbit-skeletal muscle probably possesses an active centre similar to the binding site of serum TBP. It has a higher affinity for thyroxine than any of its analogues; the enzyme can only attack free or non-protein-bound substrates and hence is strongly inhibited by thyroxine-binding proteins.

(4) Competition between the above proteins for thyroxine. Results obtained from experiments in which artificial systems of competition were set up between serum and intacellular TBP's and between thyroxine dehalogenase and serum or tissue TBP's suggest (*a*) that the diffusion of thyroid hormones from blood to tissue cells is governed by a process of simple equilibrium between free hormones and hormones bound to intra- and extra-cellular TBP's; (*b*) the rate of metabolism of thyroid hormones, and also probably the fraction available for their action, will be governed by the fraction of the hormones bound to intracellular proteins.

Aspects of the Mechanism of Action of Gastro-Intestinal Proteinases.

By W. H. Taylor. (*Department of Chemical Pathology, Ashton Street, Liverpool*)

Mucosal extracts from the rectum, colon, ileum and jejunum possess proteinase activity below pH 5 which characteristically yields symmetrical pH–activity curves with a maximum near pH 3·3, but which has no action upon egg albumin. Mucosal extracts from the stomach, pylorus and duodenum exhibit non-symmetrical pH–activity curves with two maxima, near pH 2·0 and 3·3. Only the former maximum occurs with egg albumin as substrate. Adenocarcinomata from all parts of the gut show symmetrical pH–activity curves with a maximum near pH 3·3 and fail to attack egg albumin. It seems that throughout the gut there is a mucosal proteinase with very similar properties, but that in the normal stomach, pylorus and duodenum, differentiation from a basic pattern has occurred.

Attempts to isolate two gastric enzymes, responsible, respectively, for the maxima near pH 2·0 and 3·3, and thus explaining this differentiation, have not been wholly successful (see Taylor, 1959*a*) Electrophoretic studies show, however, that crystalline pepsin and normal human gastric juice do contain two main proteinases, which each yield non-symmetrical pH–activity curves of proteolysis with two maxima. One proteinase, pyloric pepsin, arises from pyloric glands and possibly from the neck chief cells of the fundic glands. The other arises from the body chief cells of the fundic glands (Taylor, 1959*b*). Nine possible mechanisms by which a homogeneous enzyme may digest a homogeneous protein yielding a pH–activity curve with two maxima, have been investigated (Taylor, 1959*a*). The facts that egg albumin is digested differently from other proteins, that the digestion products at the two pH maxima differ, and that some synthetic substrates are split maximally near pH 2, others near pH 4 (Baker, 1954), indicate that the substrate bonds hydrolysed by these proteinases fall into two groups with respect to pH. Variations in the relative activities at the two maxima can be induced by simple manipulations of the proteinases, particularly during their preparation (Norris & Elam, 1940), and to explain the facts completely it must be postulated additionally that substrate bonds are attacked at two different active centres on the enzyme molecule. The differentiation that has taken place is therefore the acquisition by the gastric proteinases of a second active centre which is responsible for the proteolysis occurring maximally near pH 2.

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REFERENCES

Baker, L. E. (1954). *J. biol. Chem.* **211**, 700.
Norris, E. & Elam, E. (1940). *J. biol. Chem.* **134**, 443.
Taylor, W. H. (1959*a*). *Biochem. J.* **71**, 373.
Taylor, W. H. (1959*b*). *Biochem. J.* **71**, 384.

Metabolic Activities of a Peptidylphosphatide of Liver.

By E. Tria and O. Barnabei. (*Institute of General Physiology, University of Ferrara, Italy*)

A peptidylphosphatide soluble in chloroform–methanol and xylene, and insoluble in water, ethanol, diethyl ether and other solvents of lipids, has been isolated from liver lipids and partially purified (Tria, 1960). The peptide moiety constitutes about the 12% of the molecule; the lipid moiety, constituting about 88%, contains mainly phosphatides and 13% of cholesterol (Barnabei, 1960).

After acid hydrolysis, the amino-nitrogen rises about 10 times and amino acids, choline, inositol, reducing sugars, sphingosine, fatty acids, glycerol, cholesterol and phosphoric acid are set free. The peptidylphosphatide has evident antithyrotoxic properties and is metabolically very active.

The metabolic properties of the substance were studied, perfusing the isolated rat liver (Miller, Bly, Watson & Bale, 1951) with heparinized rat blood (usually 30–35 ml.), diluted with an equal volume of Krebs–Ringer bicarbonate solution, containing 60 mg. of casein hydrolysate, 10 μc of algal ^{14}C-labelled protein hydrolysate and 100 mg. of glucose. After 3 hr., the experiment was stopped, the liver was briefly perfused with cold physiological saline, homogenized with cold 10% trichloroacetic acid and washed as usual. The peptidylphosphatide was extracted with chloroform–methanol–hydrochloric acid (200:100:1), by a procedure similar to that described by Huggins & Cohn (1959); the solution was concentrated to dryness, plated and counted in a thin-end-window Geiger-Müller counter. The mean radioactivity was 125 counts/min./mg. of the peptide moiety for the peptidylphosphatide, and 37 counts/min./mg. for the proteins of the defatted liver.

The effect of some substances on the incorporation of labelled amino acids in the peptidylphosphatide has been studied and the significance of the findings discussed, in relation to the work of Hendler (1959) and Haining, Fukui & Axelrod (1960) about the possible role of lipids in the synthesis of proteins.

REFERENCES

Barnabei, O. (1960). *Arch. Sci. biol., Napoli*, (in the Press).
Haining, J. L., Fukui, T. & Axelrod, B. (1960). *J. biol. Chem.* **235**, 160.
Hendler, R. W. (1959). *J. biol. Chem.* **234**, 1466.
Huggins, C. G. & Cohn, D. V. (1959). *J. biol. Chem.* **234**, 257.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Tria, E. (1960). *L. Ric. sci.* (in the Press).

Metabolism of DL-[2-^{14}C]Lysine in Turkey Poults.

By PRAN VOHRA and F. H. KRATZER. (*Department of Poultry Husbandry, University of California, Davis, California, U.S.A.*)

DL-[2-^{14}C]Lysine was injected subcutaneously into turkey poults to study its metabolism. Maximal radioactivity in respiratory carbon dioxide was reached in the second hour after injection and about 22% of the activity was recovered in carbon dioxide within 24 hr. The following tissues of poults were examined and found to have ^{14}C distributed in them: feathers, skin, kidney, spleen, liver, adrenals, lungs, heart, blood and muscles. The specific activity per mg. of tissue was highest in spleen and in kidney. Amino acids were separated from liver hydrolysates by column chromatography on Dowex-50 ion-exchange resin. The ^{14}C from lysine was found to be incorporated into glutamic acid, aspartic acid-serine, glycine, alanine, proline and arginine. Pipecolic acid and uric acid isolated from excreta of poults which received multi-injections of labelled-lysine were also radioactive. As much as 62% of radioactivity in the uric acid was elsewhere than in C-6, which arises from CO_2. The radioactivity in the rest of the molecule arises from glycine and formate. Fat and glycogen isolated from livers of injected poults were radioactive. In their studies with rats, Rothstein & Miller (1954) have postulated that radioactivity from lysine is incorporated into glucose through formate, and have also proposed a scheme for lysine metabolism in that species. The isolation of radioactive glutamic acid and pipecolic acid as products of lysine metabolism suggests that this scheme may be applicable in case of turkey poults also. The melanin granules isolated from poult feathers were radioactive.

REFERENCE

Rothstein, M. & Miller, L. L. (1954). *J. biol. Chem.* **206**, 243.

Androgens as Activators of the Pyridine Nucleotide Transhydrogenase in Human and Rat Liver.

By D. C. WILLIAMS,* D. N. BARON and MARION B. R. GORE. (*Department of Chemical Pathology, Royal Free Hospital and School of Medicine. London, W.C.* 1)

The discovery by Villee and his co-workers of a soluble enzyme system which was stimulated by oestradiol-17β in catalytic quantities led to further work by Talalay & Williams-Ashman (1958) which showed that the rate of reaction depended on an oestradiol-activated pyridine nucleotide transhydrogenase.

$$\text{TPNH} + \text{DPN} \xrightarrow{\text{transhydrogenase}} \text{DPNH} + \text{TPN}$$

(TPNH = reduced triphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide).

Hurlock & Talalay (1958) demonstrated an androsterone-activated pyridine nucleotide transhydrogenase in rat liver and Hagerman & Villee (1959) a testosterone-activated system in guinea-pig liver. We have previously shown an androgen-activated transhydrogenase system in both human and rat

prostate (Baron, Gore & Williams, 1960) and the present work demonstrates a similar system in human and rat liver.

Cell-free fractions obtained by ultracentrifugation of fresh, homogenized liver were tested for transhydrogenase activity by means of micromodifications of the methods of Hurlock & Talalay (1958) using either the acetylpyridine analogue of DPN or the glucose 6-phosphate dehydrogenase-coupled transhydrogenation reaction. Rat livers were usually obtained from freshly killed animals, but in most cases human liver was frozen to $-75°$ immediately on receipt, and stored for 1–2 hr. before homogenization. The optimum experimental conditions have been established for the measurement of steroid activation of transhydrogenase in human- and rat-liver tissues. The degree of activation of the transhydrogenase by androsterone has been investigated both in malignant and normal human liver and in the livers of intact and castrated rats. The activating effect of various androgens on the systems has been investigated and an inhibition by oestrogenic substances on this androgenic activation has been established. Human-liver transhydrogenase can be activated by the same steroids as the rat-liver enzyme but the effect is less marked. This is probably due to the varying quantities of endogenous steroid in the tissues, since the transhydrogenase is best demonstrated in livers from immature or castrated rats.

We should like to thank the British Empire Cancer Campaign for generous financial support and surgical colleagues for providing human specimens.

REFERENCES

Baron, D. N., Gore, Marion B. R. & Williams, D. C. (1960). *Biochem. J.* **74**, 20P.
Hagerman, D. D. & Villee, C. A. (1959). *J. biol. Chem.* **234**, 2031.
Hurlock, B. & Talalay, P. (1958). *J. biol. Chem.* **233**, 886.
Talalay, P. & Williams-Ashman, H. G. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 15.

Properties of λ-Endolysin, the lytic Enzyme in Lysates of Lysogenic *Escherichia coli* (λ).

By Elizabeth Work.*† (*Institut Pasteur, Paris, France*)

The bacteriolytic enzyme (λ-endolysin) in lysates of a defective lysogenic strain (P_{32}) of *E. coli* B (λ) (Jacob & Fuerst, 1958) has been investigated. Induction of strain P_{32} by ultraviolet light or nitrogen mustard produced highly active lysates containing relatively few bacteriophage. Lytic activity was measured by observing changes in extinction of standard suspensions of acetone-dried cells (Work, 1959) in 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 7·0, containing mM-ethylenediaminetetra-acetic acid (EDTA). Bacterial cells were only lysed by the enzyme if harvested during exponential growth and then given a preliminary treatment with chloroform or acetone drying: older cultures were seldom susceptible. Crude lysates were active against treated cells of *E. coli* (all strains), *Salmonella typhimurium*, *Staphylococcus aureus*, *B. megaterium*; but not against *B. cereus* or *Micrococcus lysodeikticus*. The age of cultures of *B. megaterium* was much more critical than in the case of *E. coli*; at later stages of exponential growth the cells were scarcely lysed although they were still fully susceptible to lysozyme.

Endolysin was partially purified by ammonium sulphate fractionation of lysates of cultures grown in glucose–salts–amino acid media containing mM-nitrogen mustard. The enzyme had to be stabilized at all stages by mM-EDTA. Concentrate containing 2·3 μg. of protein (based on ultraviolet absorption measurements) produced 25% lysis of 0·5 mg. of dry *E. coli* B in 9 min.; 2·5 μg. (by weight) of lysozyme produced the same effect. The same amount of lysozyme was required for lysis of *B. megaterium*, but about 10 times as much endolysin.

Cell walls of *B. megaterium* were completely lysed by endolysin (Work & Lecadet, 1960), and were used as substrate to examine its properties. The optimum pH was about 7·0. Slight activation was produced by mM-EDTA, 10 mM-NaCl or 10 mM-KCl. Phosphate and certain heavy metal ions caused inhibition, the amounts giving approximately 50% inhibition (in the presence of 10 mM-NaCl) being KH_2PO_4, mM, Zn^{2+}, 0·5 mM, Fe^{2+}, 0·01 mM, Co^{2+} and Mn^{2+} had no effect at mM-concn. Co^{2+} 0·1 mM stimulated slightly, low concentrations of Zn^{2+} had no effect.

Many of these properties show that λ-endolysin differs from lysozyme; it is probably identical to the lytic enzyme of disrupted mature bacteriophage (K. Fisher, personal communication).

A Medical Research Council Exchange Fellowship and a Mrs John Jaffee Donation from The Royal Society supported the author during this work.

REFERENCES

Jacob, F. & Fuerst, C. R. (1958). *J. gen. Microbiol.*, **18**, 518.
Work, E. (1959). *Ann. Inst. Pasteur*, **96**, 468.
Work, E. & Lecadet, M. (1960). *Biochem. J.* , 39P.

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Digestion of Cell Walls of *Escherichia coli* and *Bacillus megaterium* by λ-Endolysin or Lysozyme.

By ELIZABETH WORK*† and MARGUERITE LECADET.
(*Institut Pasteur, Paris, France*)

Cell walls of bacteria contain mucopeptide components which have few amino acids and which are the substrate for bacteriolytic enzymes. Identical samples of cell walls of either *E. coli* B or *B. megaterium* were digested with either λ-endolysin concentrate (Work, 1960) or lysozyme. The soluble digests were dialysed, and the two fractions treated with fluorodinitrobenzene. Further separations were carried out on dialysates by paper chromatography and on non-dialysable fractions by paper electrophoresis. The resulting yellow spots were each hydrolysed and examined qualitatively for free and dinitrophenylated (DNP) amino acids. The amino sugars, glucosamine and muramic acid, were partly destroyed by hydrolysis.

The two enzymes produced apparently identical fragments from *B. megaterium* and solubilized the walls completely. Several spots in either fraction contained only alanine and glutamic acid, and no identifiable DNP-amino acids; other spots also contained several DNP-amino acids including the mono-DNP derivative of diaminopimelic acid (DAP). Free DAP was found only in the hydrolysates from some of the non-dialysable fragments. Walls from *E. coli* were not completely solubilized and the two enzymes did not produce identical fragments; some of the mucopeptides were not digested but lipids were found in all fractions. The amino acids of the different dialysable fragments varied in number, from one (glutamic acid) to twelve. DAP and lysine occurred together only in obviously inhomogeneous spots; glutamic acid was invariably present, aspartic acid, serine, glycine and alanine, sometimes; all were present either bound or as the DNP-derivatives. Valine, leucine and arginine (non-wall amino acids) were also sometimes found. Each non-dialysable fragment usually contained more amino acids than the dialysable fragments, including both DAP and lysine, but had no 'non-wall' amino acids.

Fragments from walls treated with fluorodinitrobenzene before enzymic digestion differed from those of untreated walls. After whole or phenol-fractionated DNP-walls had been exhaustively digested by one enzyme, treatment of the residue with the other enzyme resulted in further solubilization of mucopeptides.

A Medical Research Council Exchange Fellowship and a Mrs John Jaffe Donation from The Royal Society supported one author (E. W.) during this work.

REFERENCE

Work, E. (1960). *Biochem. J.* **76**, 38P.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **395th Meeting** *of the Society was held in the Department of Biochemistry, University of Cambridge, on Wednesday and Thursday,* 29 *and* 30 *June* 1960, *when the following papers were read:*

COMMUNICATIONS

WEDNESDAY MORNING, 29 JUNE
(ANATOMY THEATRE)

(1) A Sensitive Method for the Selective Estimation of Long-Chain Acyl Hydroxamates.

By G. H. SLOANE-STANLEY. (*Biochemistry Division, Physiology Department, Royal College of Surgeons of England, London, W.C.* 2)

The ferric hydroxamate method is extremely valuable for the estimation of acyl phosphates, esters and thiolesters; but it is somewhat insensitive, since ferric hydroxamates are not very strongly coloured. The ferric salts of the long-chain acyl hydroxamates can, however, be separated from excess of inorganic Fe^{3+} in a two-phase system; the iron combined with the hydroxamates can then be estimated by more sensitive methods.

A successful procedure is as follows: the hydroxamates are formed from esters by treating an ethanolic solution (0·4 ml.) of the lipids with 2M-$NH_2OH \cdot HClO_4$ (0·1 ml.) and 3·5N-NaOH (0·1 ml.) [cf. Stern & Shapiro (1953); Rapport & Alonzo's (1955) method has given somewhat erratic results]; this solution is mixed with 4·1 ml. of 0·097M-$Fe(ClO_4)_3$ in water–ethanol (15:4, v/v) containing 0·051N-$HClO_4$, and 3·1 ml. of tetrachloroethane, and centrifuged. The upper phase is removed and 3 ml. of the lower phase is treated with 0·01 ml. of 1·2N-$HClO_4$ and 0·5 ml. of methanolic 2M-NH_4SCN; after 1–3 hr. the extinction is read at 490 mμ in a $\frac{1}{2}$ in.-diam. tube, and is about 2·2/μequiv. of original ester, which corresponds to about 0·52 μ-atoms of iron. The results are considerably altered by changing the proportions of the reagents (particularly ethanol). The use of perchlorates throughout is desirable; less satisfactory results can be obtained with nitrates, using caprylol for extraction, but chlorides and sulphates must be avoided, since $FeCl_3$ and $Fe_2(SO_4)_3$ are too soluble in organic solvents to give reasonable blanks.

The two-phase method seems at present less accurate than Stern & Shapiro's (1953) method, but is much more sensitive; the homogeneous method gives both lower readings (E_{520} is approx. 0·36/μequiv of ester) and higher blanks (0·10–0·12 as against 0·01–0·03). The two-phase system is, moreover, specific for the long-chain acyl hydroxamates; those with 8 or fewer C atoms are not detected, while those with 14 or more are completely extracted.

Complex lipid mixtures sometimes give trouble, owing to the presence of iron-carrying substances other than hydroxamates, such as sulphatides and acidic lysoplasmalogens. If not excessive, this problem can be dealt with by, for instance, preliminary saponification to provide a 'blank', by gentle acid hydrolysis (plasmalogens) and/or by testing for iron-transport without prior formation of hydroxamates.

REFERENCES

Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 193.
Stern, I. & Shapiro, B. (1953). *Brit. J. clin. Path.* **6**, 158.

(2) Studies of the Biosynthesis of Brain Inositol Phosphatide.

By K. P. STRICKLAND, W. THOMPSON, D. SUBRAHMANYAM and R. J. ROSSITER. (*Department of Biochemistry, University of Western Ontario, London, Canada*)

Argranoff, Bradley & Brady (1958) reported that optimum incorporation of [^{3}H]inositol into inositol phosphatide of guinea-pig kidney mitochondria required the addition of both cytidine diphosphate choline (CMP-PC) and phosphatidic acid. It was proposed that cytidine diphosphate diglyceride (CMP-P-diglyceride) was formed as an intermediate in this incorporation. Paulus & Kennedy (1958, 1959) showed that the incorporation of L-α-glycerophosphate into liver inositol phosphatide specifically required cytidine triphosphate (CTP) and that CMP-P-diglyceride was probably an intermediate.

It has been found that CTP is the most effective nucleotide in stimulating the incorporation of [^{3}H]-inositol into the phosphatides of water homogenates of rat brain. The addition of phosphatidic acid in combination with CTP, but not with CMP-PC, further stimulated this incorporation. Synthetic D-αβ-diglycerides were ineffective compared to the corresponding phosphatidic acids in this system.

After incubation of [^{14}C]phosphatidic acid (prepared by the enzymic degradation of [^{14}C]lecithin

isolated from liver slices incubated with [1-^{14}C]-glycerol) with brain homogenates, radioactivity was located in the hydrolysis product IP derived from inositol phosphatide obtained by the method of Dawson (1954). CTP greatly stimulated the labelling of IP while CMP-PC was relatively ineffective.

Brain homogenates supplemented with adenosine triphosphate and coenzyme A, catalysed the incorporation of radioactivity from L-α-glycerophosphate labelled with ^{32}P or ^{14}C into IP and the hydrolysis product GP, derived from phosphatidic acid. The labelling of IP was greatly increased by CMP-PC while CTP caused only a slight increase. In the presence of either cytidine nucleotide the labelling of phosphatidic acid was decreased. Under these conditions the specific activity of IP greatly exceeded that of the GP derived from phosphatidic acid.

The inositol phosphatide labelled from [^{3}H]-inositol or L-α-[^{32}P]glycerophosphate was chromatographically similar to liver phosphatidyl inositol and on hydrolysis in 6N-HCl yielded a product which was electrophoretically identical to inositol monophosphate.

These findings indicate that phosphatidic acid can act as a precursor of brain inositol phosphatide in a mechanism that requires CTP as a cofactor. This pathway may involve CMP-P-diglyceride as an intermediate. On the other hand, L-α-glycerophosphate is incorporated into the inositol phosphatide of brain in a series of reactions that presumably involve fatty acyl coenzyme A and CMP-PC. Phosphatidic acid may not be an intermediate in this mechanism.

REFERENCES

Argranoff, B. W., Bradley, R. M. & Brady, R. O. (1958). *J. biol. Chem.* **233**, 1077.
Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Paulus, H. & Kennedy, E. P. (1958). *J. Amer. chem. Soc.* **80**, 6689.
Paulus, H. & Kennedy, E. P. (1959). *J. Amer. chem. Soc.* **81**, 4436.

(3) The Hydrolysis Products of Triphosphoinositide B Prepared from Ox Brain.

By R. M. C. Dawson and J. C. Dittmer.* (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

The isolation from brain tissue of a new inositol-containing phospholipid designated triphosphoinositide B has recently been reported (Dittmer & Dawson, 1960). This is normally tightly attached to brain proteins in the form of a complex which is not extractable by neutral lipid solvents (Lebaron & Folch, 1956). The new phospholipid, prepared as a water- and chloroform-soluble sodium salt, is virtually nitrogen-free, and over 95% migrates as a single component on chromatography on formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959). The products of mild chemical hydrolysis of triphosphoinositide B have been separated using the chromatographic and ionophoretic system recently devised for the examination of phospholipid hydrolysates (Dawson, 1960). Spots were located either by spraying with indicator (bromocresol green in ethanol) or by using a modified form of the Rorem (1959) reagent. They were then eluted chromatographically with 0·1N-HCl and analysed for phosphorus, glycerol and inositol.

After a brief acid hydrolysis (7 min., 5N-HCl, 100°) the phosphorus of triphosphoinositide B was liberated in the form of inositol triphosphate (77–81%), inositol diphosphate (15–16·5%) and glycerophosphate (4–5·5%), along with trace amounts of inorganic phosphate and inositol monophosphate. Prolonging the hydrolysis for 1–3 hr. resulted in an increase in the inositol diphosphate and monophosphate and inorganic phosphate at the expense of inositol triphosphate; the glycerophosphate level did not change appreciably. Isolated inositol triphosphate slowly broke down on acid hydrolysis to give inositol diphosphate and inorganic phosphate. The portion of triphosphoinositide remaining soluble in lipid solvents after a brief acid hydrolysate was fractionated on a silicic acid column and diglyceride isolated in substantial yield.

Milk alkaline hydrolysis of triphosphoinositide B gave three main products, which were separated by paper ionophoresis and analysed. The major product was a glycerylinositol triphosphate which released glycerophosphate and inositol di- and triphosphate on subsequent acid hydrolysis. During the alkaline hydrolysis lesser amounts of substances analysing as inositol triphosphate and inositol diphosphate were also formed.

The evidence suggests therefore that in the triphosphoinositide B structure, (1) three phosphate moieties are esterified to each inositol ring and (2) the glycerols are each esterified with two fatty acids and the vacant hydroxyl group esterified with a phosphate moiety attached to inositol.

REFERENCES

Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
Dittmer, J. C. & Dawson, R. M. C. (1960). *Biochim. biophys. Acta*, **40**, 379.
Hörhammer, L., Wagner, H. & Richter, G. (1959). *Biochem. Z.* **331**, 155.
Lebaron, F. N. & Folch, J. (1956). *J. Neurochem.* **1**, 101.
Rorem, E. S. (1959). *Nature, Lond.*, **183**, 1739.

* U.S. Public Health Service Research Fellow of the National Heart Institute.

(4) The Hydrolysis of Ox-Brain Diphosphoinositide Fractions.

By R. B. Ellis, P. Kemp and J. N. Hawthorne.
(*Department of Medical Biochemistry and Pharmacology, Medical School, Birmingham* 15)

It has been suggested that brain diphosphoinositide has a structure in which inositol '*meta*'-diphosphate (Folch, 1949) residues are linked by β-monoglyceride (Hawthorne, 1955; Folch & LeBaron, 1956). Results from the hydrolysis of ox-brain diphosphoinositide, purified by the method of Folch (1949), are difficult to reconcile with this structure and show that the lipid preparation contains more than one component.

The molar proportions of inositol, carboxylic ester, glycerol and phosphoric acid in the diphosphoinositide preparation were, respectively, 1:2:1:2. The ester result differs from that of Folch (1949), who obtained a ratio fatty acid:P of 1:2. Hawthorne & Chargaff (1954) found this ratio to be 1·1:1. In the present study, mild acid hydrolysis released diglyceride corresponding to roughly 20% of the glycerol present.

The phosphates released by mild alkaline hydrolysis were chromatographed on Dowex-1 columns by the method of Hawthorne & Hübscher (1959). Glycerylphosphorylinositol was detected in amounts corresponding to roughly 6% of the original lipid P. This ester is probably derived from phosphatidylinositol. Hörhammer, Wagner & Hölzl (1960) have reported the occurrence of this phospholipid in brain.

Three other phosphates, *A*, *B* and *C*, were eluted from the columns, though it is not yet clear whether these are all pure compounds. *A* and *B* were eluted with 0·1M-formic acid–0·4M-ammonium formate. Their column behaviour suggests that they are inositol diphosphates. No bound glycerol was detected in either substance. Compound *C* accounted for more than half the P applied to the column, and was eluted with M-sodium chloride. It appears to be a higher phosphate of inositol than the diphosphate.

The diphosphoinositide was also hydrolysed by a liver enzyme prepared by the method of Kemp, Hübscher & Hawthorne (1959). Ion-exchange chromatography showed very little inositol monophosphate, glycerophosphate or inorganic P in the hydrolysate. Peaks *A*, *B* and *C* were again seen, and in similar relative quantities to those found after alkaline hydrolysis.

P. Kemp is in receipt of a D.S.I.R. studentship.

REFERENCES

Folch, J. (1949). *J. biol. Chem.* **177**, 497.
Folch, J. & LeBaron, F. N. (1956). *Canad. J. Biochem. Physiol.* **34**, 305.
Hawthorne, J. N. (1955). *Biochim. biophys. Acta*, **18**, 389.
Hawthorne, J. N. & Chargaff, E. (1954). *J. biol. Chem.* **206**, 27.
Hawthorne, J. N. & Hübscher, G. (1959). *Biochem. J.* **71**, 195.
Hörhammer, L., Wagner, H. & Hölzl, J. (1960). *Biochem. Z.* **332**, 269.
Kemp, P., Hübscher, G. & Hawthorne, J. N. (1959). *Biochim. biophys. Acta*, **31**, 585.

(5) Observations on Hydroxyindole Oxidases.

By W. G. Levine (introduced by H. Blaschko).
(*Department of Pharmacology, University of Oxford*)

It has recently been shown that the gill plates of *Mytilus edulis* contain a cyanide-sensitive enzyme that oxidizes 5-hydroxyindoles (5-hydroxytryptophan, 5-hydroxytryptamine and bufotenine) with the formation of brown pigments (Blaschko & Milton, 1960). Preparations of gill plates act also on 4-hydroxyindoles (e.g. psilocine) and 6-hydroxyindoles (Blaschko & Levine, 1960).

Since it is known that caeruloplasmin also oxidizes 5-hydroxytryptamine (Porter, Titus, Sanders & Smith, 1957), a comparison has now been made between caeruloplasmin of pig plasma and the *Mytilus* enzyme. It was found that the mammalian enzyme acted not only on bufotenine, but also on its 4-hydroxy and 6-hydroxy analogues. The former is psilocine (Hofmann & Troxler, 1959); it is oxidized with the formation of a deep-blue compound; this is analogous to what occurs when psilocine is oxidized by the molluscan enzyme. Caeruloplasmin does not act on 5-hydroxytryptophan (Martin, Eriksen & Benditt, 1958) and 5-hydroxyindole, whereas the gill plate oxidase acts on these two compounds almost as rapidly as on 5-hydroxytryptamine.

These observations show that the substrate specificity of these two enzymes is not restricted to indoles carrying a hydroxyl group at C-5.

REFERENCES

Blaschko, H. & Levine, W. G. (1960). *Biochem. Pharmacol.* **2**, 168.
Blaschko, H. & Milton, A. S. (1960). *Brit. J. Pharmacol.* **15**, 42.
Hofmann, A. & Troxler, F. (1959). *Experientia*, **15**, 101.
Martin, G., Eriksen, N. & Benditt, E. P. (1958). *Fed. Proc.* **17**, 447.
Porter, C. C., Titus, D. C., Sanders, B. E. & Smith, E. V. C. (1957). *Science*, **126**, 1014.

(6) The Estimation of Monoamine Oxidase.

By A. L. Green and Thelma M. Haughton. (*Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts*)

The reaction catalysed by monoamine oxidase is generally represented (e.g. Zeller, 1951) by

$$R{\cdot}CH_2{\cdot}CH_2{\cdot}NH_2 + H_2O + O_2 \rightarrow R{\cdot}CH_2{\cdot}CHO + NH_3 + H_2O_2.$$

The enzymic activity is most often determined from the oxygen uptake, but this is relatively insensitive, while with crude enzyme preparations the oxygen consumption per mole of substrate decomposed can be very variable (Cotzias & Greenough, 1959). The peroxide is too rapidly destroyed by catalase present in normal monoamine oxidase preparations to be of value for estimation, while measurement of the ammonia liberated, although sensitive, is laborious. Measurement of substrate disappearance is unsatisfactory for kinetic studies where it is desirable to maintain approximately constant substrate concentration.

Measurement of the aldehyde formation is hindered by the ease with which further oxidation can occur, but provided semicarbazide is present in the assay mixture, this further oxidation can be suppressed, and the aldehyde semicarbazone can subsequently be converted into the 2:4-dinitrophenylhydrazone (Richter, 1937). The colour of the dinitrophenylhydrazone in alkali provides a sensitive means of assay. The assay mixture contains enzyme, buffer, semicarbazide and tyramine, which are shaken in air. The reaction is terminated with acid and the mixture is deproteinized by boiling and centrifuging. 2:4-Dinitrophenylhydrazine is added and the dinitrophenylhydrazone formed is extracted first into benzene and then into alkali. The extinction of the alkaline solution at 450 mμ provides a direct measure of the enzymic activity. Under our conditions an extinction of 0·1 corresponds to an oxygen uptake of about 2 μl.

A further assay method investigated was the replacement of oxygen as a hydrogen acceptor by tetrazolium salts (Lagnado & Sourkes, 1956). On reduction, these compounds give formazans whose intense colour, when extracted into butanol, provides a measure of the enzymic activity. The non-linear dependence of extinction on enzyme concentration, found when the reaction was performed in air, could be overcome by use of anaerobic conditions. However, when no oxygen was present reduction was almost as rapid in the absence as in the presence of substrate. This non-specific reduction could not be suppressed by monoamine oxidase inhibitors, nor by ethylenediaminetetra-acetic acid, iodoacetate, phenylmercuric acetate, malonate, azide, cyanide or fluoride ions.

REFERENCES

Cotzias, G. C. & Greenough, J. J. (1959). *Nature, Lond.*, **183**, 1732.
Lagnado, J. R. & Sourkes, T. L. (1956). *Canad. J. Biochem. Physiol.* **34**, 1095.
Richter, D. (1937). *Biochem. J.* **31**, 2022.
Zeller, E. A. (1951). In *The Enzymes, Chemistry and Mechanism of Action*, vol. 2, part 1, p. 536. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

(7) Further Purification and Properties of the Amine Oxidase of Pea Seedlings.

By P. J. G. Mann. (*Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts*)

The amine oxidase of pea seedlings, like the diamine oxidase of animal tissues, is inhibited by carbonyl reagents; this led to the suggestion that the prosthetic group contains pyridoxal phosphate. Evidence that the prosthetic group contains both pyridoxal phosphate and flavinadenine dinucleotide was reported by Goryachenkova (1956). The enzyme is inhibited by sodium diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthate (Mann, 1955) and also by 8-hydroxyquinoline and dithizone (Werle & Hartung, 1956) and by 2:2′-dipyridyl and 1:10-phenanthroline (Suzuki, 1959); this suggests it is a metalloprotein.

Preparations of the amine oxidase obtained by the method of Mann (1955) have now been further purified two- to three-fold by column chromatography on calcium phosphate (hydroxyapatite) and aminoethyl- or diethylaminoethyl-cellulose. The purified preparations were pink. The absorption spectrum over the range 300–700 mμ showed only one band with maximum absorption at about 500 mμ. The pink colour was discharged by dithionite and restored by oxygenation. These changes were accompanied by disappearance and reappearance of the band with maximum at 500 mμ. When 1:4-diaminobutane was added to the enzyme solution, under anaerobic conditions, the colour of the reaction mixture changed from pink to yellow. The band with maximum at 500 mμ disappeared and new bands appeared with maxima at 350, 435 and 465 mμ. On oxygenation the pink colour was restored quickly and the absorption spectrum reverted to that of the untreated enzyme. When hydrazine was added to the enzyme the pink colour changed to yellow, the band with maximum at 500 mμ disappeared and new bands appeared with maxima at 335 and at about 420 mμ.

Preliminary results of an investigation of the heavy-metal content showed that the copper content of the preparations increased during the

chromatographic procedure, reaching 0·08 % in the purest preparations. The copper was not removed by dialysis of the preparations against sodium diethyldithiocarbamate at pH 7.

It is suggested that the pink colour of the preparation is due to the enzyme, the prosthetic group of which may contain a copper complex.

REFERENCES

Goryachenkova, E. V. (1956). *Biochemistry (U.S.S.R.)*, **21**, 249.
Mann, P. J. G. (1955). *Biochem. J.* **59**, 609.
Suzuki, Y. (1959). *Naturwissenschaften*, **13**, 427.
Werle, E. & Hartung, G. (1956). *Biochem. Z.* **328**, 228.

(8) Further Exploration of the Active Centre of Monoamine Oxidases.

By E. A. Zeller, W. L. Pacha and S. Sarkar. (*Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois, U.S.A.*)

The active centre of enzymes may be differentiated into a primary part which is always involved in the formation of the enzyme–substrate complex, and around it, a secondary area which establishes bonds only with certain substrates. Previous studies on monoamine oxidases and diamine oxidases led to some conclusions concerning size, composition, optical configuration, etc., of their active centres as defined above. Monoamine oxidase was further explored by using several homologous series of substrates and inhibitors. Among the phenylalkylamines, phenylethylamine turned out to be the optimum substrate. *cis*- and *trans*-2-Phenyl*cyclo*propylamine appeared to be the best inhibitors of monoamine oxidase (Sarkar, Banerjee, Ise & Zeller, 1960) while 1-phenyl*cyclo*propylamine showed much less efficiency. Benzylhydrazine and its analogues were superior to the other homologous inhibitors. In all three cases the optimum structures displayed the same backbone, consisting of a benzene ring, a two-membered side-chain, and an amino group. When the isosterism between the $>CH_2$ and $>NH$ residues is considered the similarity becomes even greater and the hydrazines may be termed pseudoamines. In removing the benzene ring a sharp drop of all substrate and inhibitor activities was caused. It is concluded that the three classes of compounds, amines as well as pseudoamines, act in a similar fashion on the active centre of monoamine oxidase which may contain an analogous aromatic system plus a two-membered side-chain (Zeller, 1959). Hydrophobic forces come into play between the related structures of the substrate or inhibitor molecules and the enzyme and help to adjust the smaller molecules to the active centre.

With regard to α-hydrogens, one seems to be required in hydrazines (Zeller, Barsky, Fouts & Lazanas, 1955) and in arylalkylamines to produce an effective bond between monoamine oxidase and the substrate or inhibitor molecule, while a second α-hydrogen, if one is available, participates in the process of dehydrogenation. To one part of the active centre is tentatively assigned the structure —X—Ŷ—, in which X serves as an acceptor for the α-carbon, and Ŷ for a proton derived from an α-hydrogen.

REFERENCES

Sarkar, S., Banerjee, R., Ise, M. S. & Zeller, E. A. (1960). *Helv. chim. acta*, **43**, 439.
Zeller, E. A. (1959). *Pharmacol. Rev.* **11**, 387.
Zeller, E. A., Barsky, J., Fouts, J. R. & Lazanas, J. C. (1955). *Biochem. J.* **60**, v.

(9) Effect of Certain Hydrazines and Amines on Monoamine Oxidase and Some Pyridoxal Phosphate-Requiring Enzymes.

By Edith Heilbronn (introduced by W. J. Whelan). (*Research Institute of National Defence, Sundbyberg* **4**, *Sweden*)

The effect *in vitro* of the compounds, either well-known simple inhibitors of monoamine oxidase (e.g. *trans*- and *cis*-phenyl*cyclo*propylamine, iproniazide, benzylhydrazine, etc.) or derivatives of γ-aminobutyric acid or of glutamic acid, on monoamine oxidase, diamine oxidase and brain glutamic decarboxylase was studied. It was found, in agreement with other authors, that monosubstituted hydrazines as well as *NN'*-disubstituted hydrazines were inhibitors of monoamine oxidase (substrate tyramine, pI_{50} values for the compounds used between 5 and 7). The hydrazides of γ-aminobutyric acid and γ-guanidinobutyric acid did not inhibit the enzyme, nor did hydrazones inhibit. (The highest concentration of inhibitor in the final solution was 0·1 mM.) Diamine oxidase (substrate putrescine) was inhibited by the hydrazines, hydrazides and some hydrazones, while the amines did not inhibit the enzyme. None of the compounds had any inhibitory action on brain glutamic decarboxylase. Both diamine oxidase and glutamic decarboxylase are pyridoxal phosphate-depending enzymes. It seems that a reaction between the hydrazines or hydrazides and pyridoxal phosphate cannot alone be the reason for inhibition of a pyridoxal phosphate-requiring enzyme.

WEDNESDAY AFTERNOON, 29 JUNE
(ANATOMY THEATRE)

(10) Some Kinetic Observations on a New Preparation of Cytochrome Oxidase.

By Q. H. GIBSON, C. GREENWOOD and V. MASSEY.
(*Department of Biochemistry, University of Sheffield*)

A soluble cytochrome oxidase preparation has been obtained by partial digestion of the Keilin–Hartree heart-muscle preparation (Keilin & Hartree, 1939) with venom of *Agistrodon piscivorus*. After removal of insoluble material by centrifuging, the supernatant is fractionated on a calcium phosphate gel column, the haem proteins other than cytochrome oxidase remaining bound to the gel. Although the material is suitable for spectroscopic examination, it shows a marked Tyndall phenomenon, and remains in solution only at alkaline pH. The spectra of the various forms are similar to those of cholate-containing cytochrome oxidase preparations (Wainio, Eichel, Person & Cooperstein, 1950; Smith & Stotz, 1954). In spectrophotometric assays by the method of Wainio *et al.* (1950), Q_{O_2} values up to 1500 have been obtained.

Kinetic measurements have been made by combining the method of flash photolysis (see Porter & Norrish, 1954) with the regenerative stopped-flow principle of Chance (1954). In these determinations the carbon monoxide compound of the oxidase, with mM-2:3-dimercaptopropanol, was mixed in the flash apparatus of Gibson (1959) with 0·05 part of its volume of oxygen-containing buffer. On firing the flash the CO dissociates, liberating reduced cytochrome oxidase which then reacts with oxygen, the reaction being followed spectrophotometrically. The oxidized enzyme is slowly reduced by the 2:3-dimercaptopropanol, and the whole cycle may be repeated several times using the same enzyme solution. This method has the advantage of being applicable to the somewhat gelatinous cholate preparations, as well as to the venom-digested material described here. The photochemical difference spectrum of the CO compound of the venom preparation has been obtained and agrees with that reported by Chance (1953*a*) for the crude Keilin–Hartree preparation. The rate of combination with CO, both for venom and cholate preparations measured both by flash photolysis and by the stopped-flow method (Gibson, 1954) is $1{\cdot}4 \times 10^4$ M^{-1} sec.$^{-1}$ at 20°, a value similar to Chance's (1953*b*) for the crude Keilin–Hartree preparation.

The reaction with oxygen is complex, an initial rapid approximately second-order reaction being followed by changes extending over several seconds. The rate of the initial reaction (3×10^7 M^{-1} sec.$^{-1}$) has been very uniform in all the preparations both of cholate and venom types which have been examined. Titration with oxygen in the stopped-flow apparatus gives a change in extinction at 445 mμ of 80/m-mole of oxygen for the total change reduced → oxidized. The rate of the initial reaction appears to be independent of temperature.

REFERENCES

Chance, B. (1953*a*). *J. biol. Chem.* **202**, 397.
Chance, B. (1953*b*). *J. biol. Chem.* **202**, 407.
Chance, B. (1954). *Disc. Faraday Soc.* **17**, 123.
Gibson, Q. H. (1954). *Disc. Faraday Soc.* **17**, 137.
Gibson, Q. H. (1959). *Biochem. J.* **71**, 293.
Keilin, D. & Hartree, E. F. (1939). *Proc. Roy. Soc.* B, **127**, 167.
Porter, G. & Norrish, R. G. W. (1954). *Disc. Faraday Soc.* **17**, 40.
Smith, L. & Stotz, E. (1954). *J. biol. Chem.* **209**, 819.
Wainio, W. W., Eichel, B., Person, P. & Cooperstein, S. J. (1950). *J. biol. Chem.* **183**, 89.

(11) Effect of Oestradiol-17β on the Production of Reduced Triphosphopyridine Nucleotide in the Rat Uterus.

By K. MONGKOLKUL and J. K. GRANT.* (*Department of Biochemistry, University of Edinburgh*)

The importance of triphosphopyridine nucleotide in the reduced form (TPNH) in reductive biosynthesis is now generally recognized. The production of TPNH in the oestrogen-stimulated rat uterus has therefore been studied.

In the present investigation no significant increase in concentration of TPNH in the ovariectomized rat uterus could be demonstrated after administration of oestradiol-17β *in vivo*. Such an observation does not, however, preclude an increased rate of turnover of TPNH as might be indicated by increased activity of TPNH-generating systems. No evidence has been obtained that increased amounts of TPNH arise from stimulation of a pyridine nucleotide transhydrogenase by oestrogen, although this effect has been demonstrated in human endometrium and other tissues (Hagerman & Villee, 1952; Talalay & Williams-Ashman, 1958).

It has been found, however, that the glucose 6-phosphate and 6-phosphogluconic acid dehydrogenase activities of the uterus *in vitro* are markedly enhanced by administration of oestradiol-17β to castrated rats. This effect is diminished by the simultaneous administration of the cytotoxic drug DON (6-diazo-5-oxo-nor-L-leucine) which is known to inhibit uterine growth in response to oestrogen (Poulson, Robson & Wander, 1960). The activity of

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*iso*citric dehydrogenase is uninfluenced by oestrogen administration. It is therefore possible that an important effect of oestrogen in the uterus is to control glucose oxidation by the pentose phosphate shunt and thus to influence the availability of TPNH and to provide ribose 1-phosphate for nucleoside biosynthesis at the same time.

REFERENCES

Hagerman, D. D. & Villee, C. A. (1952). *Arch. Biochem. Biophys.* **40**, 481.
Poulson, E., Robson, J. M. & Wander, A. C. E. (1960). *J. Endocrin.* **19**, 359.
Talalay, P. & Williams-Ashman, H. G. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 15.

(12) Reductases, Glycolysis and Protein Content of Rat Brain after Shock With and Without Tranquilization.

By H. Ninomiya, R. W. Buxton and M. Michaelis. (*Division of Experimental Surgery, University of Maryland School of Medicine, Baltimore, Maryland, U.S.A.*)

Greig (1944*a*) had reported that destruction of cozymase and of flavin alloxazine dinucleotide occurred in organs excised from dogs while in haemorrhagic shock, and the same author (Greig, 1944*b*) assumed destruction of the apo-enzymes of lactic dehydrogenase and of amino acid oxidase in shock, as a conclusion from results obtained with tissue extracts after anoxic incubation *in vitro*. It seemed of interest to see whether shock would lead to a change in the activities of some intracellular respiratory enzymes, and, concomitantly, to alterations of metabolic patterns, such as glycolysis. In view of reports that tranquilizers and ganglionic blocking agents afford protection against shock (e.g. Beck & Alexander, 1959), the tests were also done with tranquilized animals.

Young adult Wistar rats were drummed in the Noble–Collip apparatus, 500 turns within 12·5 min.; the internal diameter of the drums was 37·4 cm. They were decapitated 2 hr. after exposure, with manifestations of shock clearly evident. Their brains were homogenized in Krebs–Ringer phosphate, pH 7·4, and were assayed for reductases, comparing them with controls. A general inhibition, statistically significant for six enzymes, was seen with lactic dehydrogenase, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) cytochrome *c* reductases, α-glycerophosphate dehydrogenase, DPN and TPN *iso*citric dehydrogenases, DPN and TPN malic dehydrogenases, and succinic dehydrogenase. The Pasteur reaction was repressed in shock. Homogenates obtained from shocked rats showed statistically highly significantly increased protein nitrogen content (Kjeldahl) when compared with controls. These differences were eliminated in rats which had been tranquilized with chlorethoxybutamoxane prior to drumming. The results found with non-tranquilized rats suggest tissue hypoxia, and are compatible with data from earlier experiments (Packer, Michaelis & Martin, 1958).

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REFERENCES

Beck, L. V. & Alexander, V. R. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 786.
Greig, M. E. (1944*a*). *J. Pharmacol.* **81**, 164.
Greig, M. E. (1944*b*). *J. Pharmacol.* **81**, 240.
Packer, L., Michaelis, M. & Martin, W. R. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 164.

(13) A Comparison Between Photosynthetic Triphosphopyridine Nucleotide Reductase from *Chlorella* and Higher Plants.

By R. Hill and Fay Bendall. (*Department of Biochemistry, University of Cambridge*)

Hill, Northcote & Davenport (1953) showed the presence of a methaemoglobin-reducing factor in *Chlorella pyrenoidosa*, similar to that found in higher plants by Davenport, Hill & Whatley (1952). Davenport (1959) showed that this methaemoglobin-reducing factor was the same as the photosynthetic pyridine nucleotide reductase separated by San Pietro & Lang (1958). We have now been able to prepare the reductase from *Chlorella* in sufficient amount to determine its spectroscopic properties, breaking the cells in an International Equipment Co. no. 6061 shaker head mounted in an International Refrigerated centrifuge, and using the purification procedure described by Davenport & Hill (1960).

A solution of reductase prepared from *Chlorella* (0·7 mg. of protein/ml.) gave the following values for log I_0/I: at 600 mμ 0·137, at 470 mμ 0·250 (shoulder), at 415 mμ 0·268 (peak) and at 396 mμ 0·280 (trough). These values may be compared with those for *Datura* sp. (1·0 mg. of protein/ml.) which gave: at 600 mμ 0·088, at 470 mμ 0·297 (shoulder), at 415 mμ 0·340 (peak) and at 396 mμ 0·322 (trough). Thus the spectrum of photosynthetic triphosphopyridine nucleotide reductase from *Chlorella* closely resembles that from the higher plants.

Reductase preparations from *Chlorella* and *Petroselenium sativum* each reduced methaemoglobin and triphosphopyridine nucleotide; when the rates of reduction were related to the values of the

extinction coefficient of the protein solution at 415 mμ they were approximately the same.

We thank Dr D. H. Northcote for the *Chlorella* used in this work. The work is part of a programme supported by the Agricultural Research Council.

REFERENCES

Davenport, H. E. (1959). *Biochem. J.* **73**, 45P.
Davenport, H. E. & Hill, R. (1960). *Biochem. J.* **74**, 493.
Davenport, H. E., Hill, R. & Whatley, F. R. (1952). *Proc. Roy. Soc.* B, **139**, 346.
Hill, R., Northcote, D. H. & Davenport, H. E. (1953). *Nature, Lond.*, **172**, 948.
San Pietro, A. & Lang, H. M. (1958). *J. biol. Chem.* **231**, 211.

(14) Some Properties of Mitochondrial D-α-Hydroxy Acid Dehydrogenase.

By P. K. TUBBS. (*Department of Biochemistry, University of Cambridge*)

Huennekens, Mahler & Nordmann (1951) and Mahler, Tomisek & Huennekens (1953) reported the oxidation of D-lactate by liver and kidney particles. A soluble enzyme catalysing this oxidation has since been obtained from mitochondria, and a preliminary report published (Tubbs & Greville, 1959). The properties of this enzyme have now been examined in more detail.

The enzyme used in this work has been obtained from acetone-dried rabbit-kidney mitochondria by extraction with slightly alkaline buffer, and has been further purified some 20-fold. It catalyses the oxidation of various D-α-hydroxy acids: D-lactate and higher straight-chain homologues, D-glycerate, D-malate, D- and *meso*-tartrates are all substrates, but glycollate and 3-phospho-D-glycerate are inert. The products from lactate and glycerate have been identified as pyruvate and hydroxypyruvate, respectively. Various substances can function as acceptors: 2:4-dichlorophenolindophenol and cytochrome *c* have been most commonly used. Phenazine methosulphate (3 μg./ml.) considerably increases the rate of reduction of both these acceptors. No added cofactors are needed for full activity, even after extensive dialysis.

Low (less than 0·5 mM) concentrations of cyanide greatly stimulate the activity of freshly prepared enzyme, while higher concentrations inhibit in an essentially competitive manner; with less fresh preparations only the inhibitory effect remains. These opposing effects of cyanide are both more marked at pH 8·5 (the routine assay pH) than at pH 7·5, suggesting that cyanide ion may be the active species.

Chelating agents, for example ethylenediaminetetra-acetate and 1:10-phenanthroline, are strong inhibitors. The inhibition is progressive, obeys first-order kinetics, and is prevented, but not reversed, by substrate (and, surprisingly, by L-lactate). Cyanide (0·2 mM) greatly potentiates the action of these inhibitors; this effect was discussed.

While a bound metal is clearly implicated in the action of the enzyme, attempts to confirm the probable presence of flavin have been unsuccessful. Activity lost on ultraviolet irradiation is not restored by flavin nucleotides, and the spectrum of the purest preparation yet obtained shows no evidence of flavin reduction by substrate.

Similar D-lactate dehydrogenase preparations have recently been described from yeast (Labeyrie, Slonimski & Naslin, 1959) and various bacteria. Possible metabolic roles (see, for example, Dickens & Williamson, 1959; Elliott, 1959) were discussed.

REFERENCES

Dickens, F. & Williamson, D. H. (1959). *Biochem. J.* **72**, 496.
Elliott, W. H. (1959). *Nature, Lond.*, **183**, 1051.
Huennekens, F. M., Mahler, H. R. & Nordmann, J. (1951). *Arch. Biochem. Biophys.* **30**, 66, 77.
Labeyrie, F., Slonimski, P. P. & Naslin, L. (1959). *Biochim. biophys. Acta*, **34**, 262.
Mahler, H. R., Tomisek, A. & Huennekens, F. M. (1953). *Exp. Cell Res.* **4**, 208.
Tubbs, P. K. & Greville, G. D. (1959). *Biochim. biophys. Acta*, **34**, 290.

(15) The L-α-Glycerophosphate Dehydrogenase Component of an Oxidase System Present in *Trypanosoma rhodesiense*.

By P. T. GRANT and J. R. SARGENT. (*Department of Biological Chemistry, University of Aberdeen*)

The major part of the respiration of the African trypanosome, *Trypanosoma rhodesiense*, can be attributed to an L-α-glycerophosphate (L-α-GP) oxidase whose reaction with oxygen does not involve pyridine nucleotides and is insensitive to cyanide, azide, antimycin *A*, and 2-heptyl-4-hydroxyquinoline *N*-oxide (Grant & Sargent, 1959, 1960).

The oxidase activity of fresh cell-free preparations, about 1 μmole of L-α-GP utilized/min./mg. of N, tended to lose activity on standing, even at 0°, and was completely inactive after freeze-drying or on treatment with acetone at −15°. The residual material, however, retained its ability to oxidize L-α-GP to dihydroxyacetone phosphate in the presence of a suitable hydrogen acceptor such as phenazine methosulphate or 2:6-dichlorophenolindophenol. Methylene blue or brilliant cresyl blue were almost inactive in this respect. The enzyme present in both acetone powders and freeze-dried preparations was stable for at least 6 months when stored at 0°.

A thiol group or groups appears to be essential for the active enzyme, since *p*-chloromercuribenzoate (μM) and aromatic arsenicals such as melarsen oxide (10 μM) decreased the activity by about 50% and this inhibition could be relieved by glutathione.

The activity of the enzyme was unaffected by 8-hydroxyquinoline (mM) when 2:6-dichlorophenol-indophenol was the acceptor. In contrast, when the auto-oxidizable compound phenazine methosulphate was used the uptake of oxygen was considerably enhanced in the presence of this chelating agent, a result similar to that observed in the assay of succinic dehydrogenase with the same acceptor (Kearney & Singer, 1956). A possible explanation of this effect is that 8-hydroxyquinoline minimizes the metal-catalysed oxidation of an essential thiol group on the enzyme by hydrogen peroxide formed during the auto-oxidation of the reduced acceptor.

Glycerol, β-glycerophosphate and propane-1:2-diol phosphate neither served as substrates for the enzyme nor did they affect the oxidation of L-α-GP. The activity of the enzyme under optimum conditions of pH, substrate and phenazine methosulphate concentrations was about 2·2 μmoles of L-α-GP utilized/min./mg. of N, which would suggest that this primary dehydrogenase component is not the rate-limiting reaction in the intact L-α-GP oxidase system.

We thank the Carnegie Trust and the Medical Research Council for generous financial support.

REFERENCES

Grant, P. T. & Sargent, J. R. (1959). *Biochem. J.* **73**, 4P.
Grant, P. T. & Sargent, J. R. (1960). *Biochem. J.* **76**, 229.
Kearney, E. B. & Singer, T. P. (1956). *J. biol. Chem.* **219**, 963.

(16) Effects of Phenylmercuric Acetate on the Enzymic Activity of L-Myosin.

By G. D. Greville and D. F. Tapley. (*Department of Biochemistry, University of Cambridge*)

When L-myosin from rabbit muscle is incubated at 25° with 0·5M-KCl, buffer pH 7·5, adenosine triphosphate (ATP), Ca^{2+} and phenylmercuric acetate (PMA), the adenosine triphosphatase activity increases at first with increasing PMA concentration, passes through a maximum and is finally strongly inhibited. If, however, the myosin is pre-incubated at 25° with PMA in absence of ATP, both in presence and absence of Ca^{2+}, the maximal stimulation is about twice as great. If ATP is present during the pre-incubation with PMA, without Ca^{2+}, the stimulation is greater at suboptimum PMA concentrations than that without ATP, but the maximum is the same. If, however, ATP and Ca^{2+} are both present during the pre-incubation, the accelerations at various concentrations of PMA have the lower values observed without pre-incubation.

So ATP and Ca^{2+} when present together reduce the stimulation produced by PMA, but neither alone has this effect. With 5 mM-$CaCl_2$, 0·2 mM-ATP is just enough to cause full reduction in stimulation. With ATP absent PMA produces complete activation very rapidly (less than 3 sec. at 25°). Pre-incubation with adenosine diphosphate or monophosphate in presence of Ca^{2+} does not reduce the stimulation by PMA.

The inosine triphosphatase activity is inhibited by PMA at all concentrations (Greville & Needham, 1955). Pre-incubation with PMA in absence of substrate does not increase this inhibition.

Probably at least two thiol groups are involved at the active centre (Kielley & Bradley, 1956), one promoting the hydrolysis of ATP and another inhibiting by interaction with the 6-amino group of the adenine (Greville & Reich, 1956; Blum, 1959). The stimulation by PMA will depend on the distribution of this substance between these thiol groups. PMA reacts preferentially with the inhibitory thiol group, and the presence of ATP together with Ca^{2+} seems to render this group less highly favoured.

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REFERENCES

Blum, J. J. (1959). *Fed. Proc.* **18**, 13.
Greville, G. D. & Needham, D. M. (1955). *Biochim. biophys. Acta*, **16**, 284.
Greville, G. D. & Reich, E. (1956). *Biochim. biophys. Acta*, **20**, 440.
Kielley, W. W. & Bradley, L. B. (1956). *J. biol. Chem.* **218**, 653.

(17) Enzymology and Comparative Biochemistry of a New Lens Peptidase.

By E. A. Zeller, Renuka Banerjee and D. Shoch. (*Departments of Biochemistry and of Ophthalmology, Northwestern University Medical School, Chicago, Illinois, U.S.A.*)

Recently we described the occurrence of a peptidase in the lens of several species which catalysed the hydrolysis of L-phenylalanine ethyl ester (Zeller & Devi, 1957; Zeller & Shoch, 1959). In most instances it was measured manometrically in a system containing bicarbonate buffer pH 6·5.

In comparing the ethyl esters of a large number of L-amino acids, the tryptophan derivative was found to be the best substrate of lens peptidase.

Various esters of the same amino acids were hydrolysed with decreasing rates in the following order: methyl, ethyl, propyl, and benzyl. Neither *N*-methyl-L-phenylalanine ethyl ester nor D-tryptophan methyl ester were attacked. The amides of L-leucine and L-phenylalanine turned out to be excellent substrates, while α-methyl-DL-phenylalanine amide did not undergo deamination.

After dialysing lens homogenates against solutions of ethylenediaminetetra-acetate the activity of lens peptidase dropped sharply. The enzyme was strongly activated by Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Mg^{2+}, and Cd^{2+}, and inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, and Cr^{2+}. No trivalent cation exerted any activating or inhibitory effect on lens peptidase.

While the data given above were obtained with experiments carried out with ox-lens homogenates, similar but quantitatively different results were found with rabbit-lens preparations. The two agents seem to belong to a group of homologous enzymes which appeared to be different from other known peptidases.

The activity of lens peptidase was significantly reduced in human senile cataract and in cataracts produced in rabbits by X-rays or by intralenticular injections of iodoacetate.

REFERENCES

Zeller, E. A. & Devi, A. (1957). *Amer. J. Ophthal.*, **44**, II, 281.
Zeller, E. A. & Schoch, D. (1959). *Fed. Proc.* **18**, 358.

(18) The Purification of Interferon.

By D. C. BURKE.* (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Interferon is the name that was given to a product of the interaction of inactivated influenza virus and host cells (Isaacs & Lindenmann, 1957). It inhibits the growth of a variety of viruses and appears to be the mediator of the phenomenon of virus interference. Since preliminary experiments suggested that interferon is a protein (Lindenmann, Burke & Isaacs, 1957), its purification was followed by concurrent determination of protein and of biological activity, i.e. estimation of its ability to interfere with influenza virus. A 20-fold purification has been achieved, to give a product which acts as a single substance by a number of criteria.

Interferon was produced by the interaction of ultraviolet-inactivated influenza virus with chick chorio-allantoic membranes (Burke & Isaacs, 1958). An initial 100-fold concentration was effected by addition of ammonium sulphate to 73% saturation. The precipitate was dissolved in Earle's buffered saline and dialysed under pressure.

This material was then chromatographed on sulphomethylcellulose at pH 2 with an increasing concentration of chloride.

The biologically active fractions were then chromatographed on diethylaminocellulose at pH 6·6 with an increasing concentration of phosphate. The active material was then examined by starch-gel electrophoresis (Smithies, 1955) at pH 8·9 and was found to contain four components, only one of which had interfering activity. The active material eluted at pH 6·6 was applied to a diethylaminoethylcellulose column at pH 4·5. The activity was not retained and the effluent was concentrated and chromatographed on diethylaminoethylcellulose at pH 5·8 with an increasing concentration of phosphate. A single symmetrical peak was obtained which gave a single band on starch-gel electrophoresis at both pH 8·9 and 2, and which gave a single component of $S_{20,w}$ 4·77 when examined in the analytical ultracentrifuge by Dr P. A. Charlwood. Molecular-weight determination by the Archibald method gave a value of 62 800. The purified preparation of interferon exhibited a typical protein ultraviolet absorption spectrum. It contained no nucleic acids and only small amounts of carbohydrate and neuraminic acid.

REFERENCES

Burke, D. C. & Isaacs, A. (1958). *Brit. J. exp. Path.* **39**, 452.
Isaacs, A. & Lindenmann, J. (1957). *Proc. Roy. Soc.* B, **147**, 258.
Lindenmann, J., Burke, D. C. & Isaacs, A. (1957). *Brit. J. exp. Path.* **38**, 551.
Smithies, O. (1955). *Biochem. J.* **61**, 629.

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WEDNESDAY AFTERNOON, 29 JUNE
(PHYSIOLOGY THEATRE)

(19) Carbohydrases in the Alimentary Tract of the Locust *Schistocerca gregaria* (Forsk.).

By W. A. L. EVANS and D. W. PAYNE. (*Department of Zoology, University College, Cardiff*)

Microqualitative tests were made by incubating small portions of gut tissue in suitable buffer-substrate solutions saturated with toluene (Evans, 1956). The results were confirmed in general by quantitative tests using dialysed freeze-dried preparations of various regions of the gut. For these tests reducing sugar was estimated colorimetrically by a modification of the Nelson (1944) and Somogyi (1945) methods. It was found unnecessary to precipitate the small quantity of protein present in the reaction mixtures and so the procedure was much more rapid.

Different regions of the alimentary tract showed the ability to hydrolyse a wide range of oligosaccharides. All the substrates containing α-glycosidic bonds were broken down by most of the tissues, namely, sucrose, maltose, αα-trehalose, melibiose, methyl α-glucoside, melezitose and raffinose. Similarly, all the substrates with β-glycosidic bonds were hydrolysed, namely, cellobiose, salicin, lactose and *o*-nitrophenyl β-D-galactoside. The enzymic degradation of these carbohydrates can be accounted for by assuming the presence of at least one of each kind of four types of enzyme: α-glucosidase, α-galactosidase, β-glucosidase and β-galactosidase.

Freeze-dried preparations of dialysed centrifuged gut contents possessed similar properties to the tissues as far as the range of substrates hydrolysed was concerned. However, the pH optima for the hydrolysis of several substrates showed a distinct shift to the acid side; e.g. sucrose and maltose from pH $5{\cdot}8 \pm 0{\cdot}05$ for tissue to pH $5{\cdot}5 \pm 0{\cdot}05$ for gut contents.

Preliminary results indicate that both gut tissue and contents contain transglycosylase activity. In maltose-reaction mixtures the appearance of isomaltose has been established by chromatographic analysis.

One of us (D.W.P.) is indebted to the D.S.I.R. for a research scholarship.

REFERENCES

Evans, W. A. L. (1956). *Exp. Parasitol.* **5**, 191.
Nelson, N. (1944). *J. biol. Chem.* **153**, 373.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.

(20) Hexose Metabolism in Maize Roots.

By V. S. Butt and H. Beevers. (*Department of Botany, University of Oxford, and Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.*)

The function of the pentose phosphate pathway in the hexose metabolism of tissues has recently attracted attention (see Holzer, 1959) and here, its operation under the action of various reducible substances has been determined from the pattern of production of $^{14}CO_2$ from maize roots fed with specifically labelled sugars. As the pentose phosphate pathway is considered to be normally inoperative in this tissue (Beevers & Gibbs, 1954), its induction can be readily recognized.

Methylene blue and *N*-methylphenazonium sulphate, both auto-oxidizable dyes which stimulated oxygen uptake and carbon dioxide production, increased the relative rate of release of C-1 from glucose and, more slowly, that of C-2. Triphenyltetrazolium chloride stimulated carbon dioxide production only, forming the characteristic red precipitate in the tissue; radiochemical evidence followed the same pattern. It suggests that the pentose phosphate pathway especially is sensitive to the oxidizing action of the dyes, presumably in their action on reduced triphosphopyridine nucleotide (TPNH); this re-oxidation must normally control the operation of the pathway. Further, when re-oxidation of TPNH is very rapid, the pathway operates as a cycle with further utilization of the hexose phosphate which it produces.

Under nitrogen, methylene blue and triphenyltetrazolium chloride were reduced, and stimulated the fermentation of glucose to carbon dioxide, though without altering the release of C-3 and C-4. The release of C-1 was increased sufficiently to account for the extra carbon dioxide. The pentose phosphate pathway will thus proceed under nitrogen if a suitable oxidant for the TPNH generated is available.

By comparison, 2:4-dinitrophenol, which induces aerobic fermentation (Beevers, 1953), increased predominantly the release of C-3 and C-4 of glucose in air; the release of carbons C-1, C-2 and C-6 followed that expected from the conventional hexose breakdown.

When nitrite was applied to the tissues, production of carbon dioxide was also stimulated (cf. Willis & Yemm, 1955). Again, the reagent stimulated primarily the release of C-1 and then C-2 from glucose, suggesting that nitrite is reduced by TPNH (Nason, Abraham & Averbach, 1954) generated in the pentose phosphate pathway, observations parallel with those of Holzer & Witt (1960) on the utilization of ammonia in yeast.

REFERENCES

Beevers, H. (1953). *Amer. J. Bot.* **40**, 91.
Beevers, H. & Gibbs, M. (1954). *Plant Physiol.* **29**, 322.
Holzer, H. (1959). *Annu. Rev. Biochem.* **28**, 171.
Holzer, H. & Witt, I. (1960). *Biochim. biophys. Acta*, **38**, 163.
Nason, A., Abraham, R. G. & Averbach, B. C. (1954). *Biochim. biophys. Acta*, **15**, 159.
Willis, A. J. & Yemm, E. W. (1955). *New Phytol.* **54**, 163.

(21) Some Effects of Chelating Agents on Roots of Tomato Seedlings.

By E. C. Cocking. (*Department of Botany, University of Nottingham*)

Ginzburg (1958) reported that 0·1 M-ethylenediaminetetra-acetic acid (EDTA) at pH 8 brought about separation of cells of the root tips of pea seedlings. It seemed of interest to re-investigate the

use of this chelating agent for the separation of plant cells as an adjunct to the study of plant proteins during growth and differentiation in roots. Investigations employing such a technique would supplement previous work based on serial sectioning of the root system (Robinson & Brown, 1952), as well as providing cell innocula for studies of proteins during the growth and differentiation of cell cultures.

Seedlings of tomato (*Lycopersicum esculentum* Mill.) were grown under aseptic conditions, and treated with sterile solutions of EDTA at 27°. It was found that mM-EDTA adjusted to pH 7·2 with N-NaOH brought about separation of the cells of the tissues of the rapidly elongating region of the root into single cells, and chains of cells, after incubation with the seedlings for 24 hr. A group of cells was isolated from among the separated cells of the apical meristem, which was probably the 'quiescent centre' (Clowes, 1956). A 6 hr. period of incubation caused the root cap to separate as a unit, exposing the apical meristematic tissue of the root. Furthermore, after treatment of the seedlings with 10 mM-EDTA at 4° for 24 hr. followed by treatment with distilled water at 27° for 6 hr. it was possible to isolate units of the stele at various stages of development and differentiation.

No separation of cells was detectable after treatment with solutions of EDTA at pH 4·5. This confirmed the conclusion of Ginzburg (1958) that separation of cells is largely dependent on the chelation by EDTA of Ca^{2+} of the middle lamella, since chelation of Ca^{2+} by EDTA is at a maximum at about pH 7·2 and at a minimum at about pH 4·5. Further evidence that alkaline earth metal chelation was involved in the separation of cells by EDTA has been obtained from investigations using *NN*-di(2-hydroxyethyl)glycine. Solutions of this chelating agent adjusted to pH 8·0 with N-HCl failed to bring about separation of cells of the root tip. This agent chelates heavy metals but has virtually no complexing activity against the alkaline earth metals (Chaberek, Courtney & Martell, 1953).

REFERENCES

Chaberek, S., Courtney, R. C. & Martell, A. E. (1953). *J. Amer. chem. Soc.* **75**, 2185.
Clowes, F. A. L. (1956). *J. exp. Bot.* **7**, 307.
Ginzburg, B. Z. (1958). *Nature, Lond.*, **181**, 398.
Robinson, E. & Brown, R. (1952). *J. exp. Bot.* **3**, 356.

(22) The Use of Tissue Cultures for the Study of Plant-Cell Walls.

By D. T. A. Lamport and D. H. Northcote. (*Department of Biochemistry, University of Cambridge*)

The rotary-shaker technique of Nickell (1956) was used to grow cell suspensions of sycamore (*Acer pseudoplatanus* L.). During the log phase the mean generation time was approx. 48 hr. and the Q_{O_2} was between 5 and 6 at 27°. Addition of coconut milk to the medium was essential for growth. An increase of coconut milk from 10 to 40% (v/v) of the medium increased the size of cell clumps from less than 10 cells per clump to several hundred. This effect of the addition of coconut milk was reversed by increasing the concentration of 2:4-dichlorophenoxyacetic acid from 0·6 to 6 p.p.m.

A thick suspension of cells in water was shaken with glass beads (2 mm. diam.) at approx. 50 cyc./sec. for 2 min. in a cell mill (Zillig & Holzel, 1958) and the cell walls were isolated by centrifuging. The walls were washed 15 times with water and a preparation with less than 1% nitrogen was obtained. Of this nitrogen approximately 13% was present as anhydrohydroxyproline. Only traces of this imino acid occurred in the protein of cell contents.

The walls were fractionated into hot-water soluble pectin 5%, pectin extracted by ethylenediaminetetra-acetic acid 10%, hemicellulose 40%, and α-cellulose 30%. The analysis resembled that of cell walls from cambial tissues (Northcote, 1958).

At least 80% of the acid phosphatase activity of the cell was present in the wall preparation. This was measured using *p*-nitrophenyl phosphate as substrate and a broad pH optimum between 6 and 6·5 was found. Inorganic phosphate was found to inhibit competitively. The rates of hydrolysis for various substrates decreased in the order: *p*-nitrophenyl phosphate; phenyl phosphate > glucose 6-phosphate > β-glycerophosphate > glucose 1-phosphate > inorganic pyrophosphate. The specific activity expressed as μmoles of *p*-nitrophenyl phosphate hydrolysed/min./mg. of nitrogen at 27° was 1·3.

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REFERENCES

Nickell, L. G. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 848.
Northcote, D. H. (1958). *Biol. Rev.* **33**, 53.
Zillig, W. & Holzel, H. (1958). *Hoppe-Seyl. Z.* **312**, 140.

(23) The Metabolism of Cinnamic Acids and Coumarins in Higher Plants.

By J. B. HARBORNE and J. JANET CORNER. (*John Innes Horticultural Institution, Hertford, Herts*)

Recently a number of workers (e.g. Pridham, 1960) have shown that simple phenols are converted into the corresponding β-glucosides when fed to plants. The fate of cinnamic acids and coumarins, compounds which may be of physiological significance to the plants in which they occur, has now been studied. Dilute aqueous solutions of these phenolic substances were either fed to leaf petioles or infiltrated into tuber disks. After 24–72 hr., the plant material was washed with water and extracted with boiling ethanol. The products were identified by means of paper chromatography and absorption spectrophotometry (Harborne, 1960). The results were compared with controls fed with distilled water.

Monohydroxy coumarins (e.g. 6- and 7-hydroxy- and 7-hydroxy-6-methoxycoumarin) are converted into the expected β-glucosides. 6:7-Dihydroxycoumarin (esculetin) is converted in some plants (e.g. potato) into an equal mixture of the 6- and 7-monoglucosides (esculin and cichoriin) and in other plants (e.g. *Clematis*) to esculin alone. Traces of the 6-diglucoside are formed in some instances. When esculin and cichoriin are fed separately, either an equal mixture of both or esculin alone are again produced.

The results of experiments with the related cinnamic acids are more varied. The main products of feeding *o*- and *p*-coumaric, caffeic, ferulic and sinapic acids are the glucose esters, rather than the expected phenolic glucosides. Even when the methyl ester of *p*-coumaric acid is fed, *p*-coumarylglucose is still the main product. Phenolic glucosides are formed as minor products in certain plants. For example, caffeic acid is metabolized by tomato leaves to give three glucose derivatives (two of which are the 3-β-glucoside and the glucose ester). Another metabolite of caffeic acid in radish leaves is esculin, and *p*-coumaric acid is converted by the same plant into caffeoylglucose.

It is concluded that cinnamic acids and coumarins undergo active turnover in plants and that plant species differ in the enzymes they contain for metabolizing them. The importance of the formation of sugar derivatives from cinnamic acids is apparent from the recent identification of a number of such compounds (e.g. *p*-coumaryl- and caffeoyl-glucose) as naturally occurring substances (Corner & Harborne, 1960, and unpublished work). Indeed, sugar esters of caffeic acid appear to occur nearly as widely as the well-known quinic esters.

REFERENCES

Corner, J. J. & Harborne, J. B. (1960). *Chem. & Ind.* p. 76.
Harborne, J. B. (1960). *Biochem. J.* **74**, 272.
Pridham, J. B. (1960). *Biochem. J.* **74**, 42P.

(24) The Presence of Sialic Acid in Human Cartilage.

By A. J. ANDERSON. (*Department of Pathology, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

Mechanical disintegration of human cartilage at the temperature of liquid nitrogen produced a finely divided product from which three fractions were isolated. Fraction *A* was extracted at pH 10·6 with a glycine buffer (0·1M, pH 11·5) followed by ethanol precipitation of the extract (63%, v/v). Fractions B_1 and B_2 were extracted at pH 11·6 from the resulting cartilage residue with 0·5N-NaOH, followed by fractional precipitation of the extract with ethanol (63 and 84%, v/v, respectively). The yields (g./100 g. of wet tissue) were: fraction *A*, 2·9; fraction B_1, 0·6; fraction B_2, 0·2. Sialic acid was detected in each fraction using a modified diphenylamine reagent (Dische, 1929). The violet chromogen absorbing at 525 mμ formed by free and bound sialic acid with this reagent was not produced with pure chondroitin sulphate, L-fucose, hexoses, glucuronic acid, α-D-glucosamine or bovine serum albumin. Mild acid hydrolysis (0·01N, 80°, 1 hr.) of fraction *A* liberated a dialysable product amounting, respectively, to 100, 10 and 12% of the sialic acid, hexose and peptide material originally present.

Protein, sialic acid, hexosamine, sulphate and total non-nitrogenous hexoses accounted for 85% of the total solids extracted. The analyses for sialic acid and hexuronic acid were, respectively: fraction *A*, 3·7, 8·6; fraction B_1, 4·1, 7·3; fraction B_2, 6·4, 0·3%. None of the 15 amino acids detected contained sulphur. Traces of hydroxyproline present in fractions *A* and B_2 indicated that collagen was not a major protein constituent.

Treatment of fraction *A* with phosphotungstic acid, trypsin, chloroform–amyl alcohol and Lloyds reagent resulted in products containing varying amounts of protein, mucopolysaccharide and sialic acid-containing components. Similar results were obtained after absorption on to benzoic acid (Anderson & Maclagan, 1955), partitioning with 50% (w/v) phenol (Courts, 1959), precipitation with 5-aminoacridine hydrochloride (Muir, 1958), hyaluronidase and papain digestion and $(NH_4)_2SO_4$ fractionation.

It would appear that the well-known difficulties attending the removal of protein material from

mucopolysaccharides apply equally to the separation of the sialic acid-containing components. Whether the sialic acid is present in the form of sialoproteins similar to those in human serum and urine or whether it is associated in some way with the linkage of mucopolysaccharides to non-collagenous peptide material, of the type first reported by Shatton & Schubert (1954), is being investigated.

REFERENCES

Anderson, A. J. & Maclagan, N. F. (1955). *Biochem. J.* **59**, 638.
Courts, A. (1959). *Biochem. J.* **73**, 600.
Dische, Z. (1929). *Mikrochemie*, **7**, 33.
Muir, H. (1958). *Biochem. J.* **69**, 195.
Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.

(25) Experiments on the Metabolism of Collagen in Lathyrism.

By E. Kulonen, Annikki Salmi and K. Juva. (*Department of Medical Chemistry, University of Turku, Turku* 3, *Finland*)

On the eighth day of hatching, 1·5 mg. of $^{15}NH_4Cl$ (10 atom % excess) was injected into the yolk sacs of chick embryos with 0·628 mg. of β-aminopropionitrile sulphate (APN). The incubation was continued 2 or 3 days, when the embryos were harvested. In one series the injection of the APN was postponed to the twelfth day and the embryos prepared on the thirteenth day. Neutral salt-soluble collagen (NSC), citrate-soluble collagen, insoluble collagen, non-protein nitrogen and in some cases 'albumin' and 'globulin' were isolated and their nitrogen analysed.

When $^{15}NH_4Cl$ and APN were administered together, the total ^{15}N-content of insoluble collagen and NSC was higher in the experimental samples than in the controls, as also was the atom % excess of ^{15}N. The ^{15}N-abundances in the NSC and in the non-protein nitrogen were of the same order; in experimental samples the atom % excess in NSC was slightly above that in the non-protein nitrogen.

When APN was injected on the twelfth day the total ^{15}N and also its abundance in the insoluble collagen decreased. The NSC increased, but ^{15}N (as atom % excess) remained almost unchanged at the level of non-protein nitrogen. The amino acid nitrogen increased but its ^{15}N-content diminished.

Corresponding experiments were made *in vitro* by incubating the carrageenin granuloma slices with [^{14}C]proline (Green & Lowther, 1959) in the presence of 10^{-3} or 10^{-2}M-APN for 4 hr. Proline and hydroxyproline were isolated from the hydrolysed protein fractions as dinitrophenyl derivatives using two-dimensional chromatography (Levy, 1954). The specific activities of dinitrophenyl-hydroxyproline were similar in both the experimental and control series, but the total activity was lower in NSC and in citrate-soluble collagen when the slices had been incubated with APN. In other experiments the NSC decreased. The specific activity of proline was higher in the supernatant, in the free-tissue amino acids and in proteins.

To conclude, *in vivo* the synthesis of collagen was enhanced by APN but the collagen became more labile metabolically. This agrees with its increased solubility (Levene & Gross, 1959; Mikkonen, Tuominen & Kulonen, 1959). The stimulation of connective tissue by APN has been reported by Bauer, Carlsson & Lindquist (1955). *In vitro*, only a decrease of the endogenous proline and of NSC was indicated.

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REFERENCES

Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1955). *Acta path. microbiol. scand.* **37**, 407.
Green, N. M. & Lowther, D. A. (1959). *Biochem. J.* **71**, 55.
Levene, C. J. & Gross, J. (1959). *J. exp. Med.* **110**, 771.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Mikkonen, L., Tuominen, T. & Kulonen, E. (1959). *Acta chem. scand.* **13**, 2125.

(26) The Reduction of Aromatic Nitro Groups During Acid Hydrolysis in the Presence of Carbohydrates and its Bearing on the Colorimetric Determination of ε-Dinitrophenyllysine.

By V. Handwerck, E. Bujard and J. Mauron (introduced by K. J. Carpenter). (*Research Laboratory of Nestlé's Products, Vevey, Switzerland*)

The determination of free ε-amino groups in proteins of foodstuffs with fluorodinitrobenzene (FDNB) involves the acid hydrolysis of the dinitrophenylated protein in presence of large amounts of carbohydrate. It has been reported (Fraenkel-Conrat, 1955) that exceptional destruction of dinitrophenyl (DNP) amino acids is associated with the presence of reducing agents. We investigated, therefore, the destruction of ε-DNP-lysine during refluxing with 100 ml. of 6N-HCl for 24 hr., in presence of various carbohydrates.

ε-DNP-lysine was measured by the method of Bruno & Carpenter (1957) for determining 'available lysine' in foods. Hydrolysis of 20 mg. of ε-DNP-lysine alone, with 140 mg. of casein, 190 mg. of mannitol, 190 mg. of gluconic acid or 190 mg. of sorbitol leads to insignificant (less than 8%)

destruction. Hydrolysis of ε-DNP-lysine with 9·5 times its weight of glucose, fructose or D-glucuronic acid lactone results in a destruction of 50, 54 and 79%, respectively, whereas with lactose, sucrose or starch the destruction is 65, 57 and 50%, respectively. Doubling the amount of starch increases the destruction to 68%. Similar results were obtained with 2:4-dinitrophenol as model substance. It is concluded that destruction is associated with carbohydrates having a reducing power or being capable of yielding reducing compounds on hydrolysis.

Reduction of nitro groups could be demonstrated with 2:4-dinitro-phenol and -toluene and ε-DNP-lysine. After refluxing these compounds with glucose or lactose in 6N-HCl, amino derivatives were separated by paper chromatography and detected by diazotization and coupling with *N*-1-naphthylethylenediamine. 2:4-Dinitrophenol yielded 2-amino-4-nitro-phenol, 4-amino-2-nitrophenol and some 2:4-diaminophenol. 2:4-Dinitrotoluene produced aminonitrotoluene and very probably diaminotoluene.

The destruction of ε-DNP-lysine observed would render the determination of 'available lysine' in foods impossible, were it not for the protective effect brought forth by the initial treatment with FDNB. Thus, treatment of milk with FDNB, removal of excess of FDNB and subsequent acid hydrolysis yields a nearly theoretical amount of ε-DNP-lysine, whereas the hydrolysis of synthetic ε-DNP-lysine with the same proportion of milk results in a destruction of more than 50%. This unexplained deactivation of carbohydrates by pretreatment with FDNB is being investigated.

REFERENCES

Bruno, D. & Carpenter, K. J. (1957). *Biochem. J.* **67**, 13P.
Fraenkel-Conrat, H. (1955). *Meth. Biochem. Anal.*, vol. 2, p. 369.

(27) Starch-Gel Electrophoresis Applied to the Detection of Hybrid Haemoglobins.

By D. B. Gammack, E. R. Huehns, H. Lehmann and E. M. Shooter. (*Department of Biochemistry, University College, London, W.C. 1, and Department of Pathology, St Bartholomew's Hospital, London, E.C. 1*)

Itano, Singer & Robinson (1959) have shown that hybrid haemoglobins may be formed when two different haemoglobins are together dissociated at acid pH and subsequently recombined by neutralization of the acid solution. Such hybrid molecules are of the parent type if the two haemoglobins have a common pair of α- or β-chains, but if the haemoglobins are singly abnormal and differ from each other in both α- and β-chains then haemoglobin A and a doubly abnormal haemoglobin are formed as well as the parent types. The hybrids which differed in net charge from the parent types were originally detected by moving-boundary electrophoresis (Itano & Robinson, 1959), or column chromatography (Vinograd, Hutchinson & Schroeder, 1959). The separation and detection of such hybrid haemoglobins has now been achieved, both in starch gel using either phosphate buffer or the discontinuous system described by Poulik (1957). The combination of the starch-gel technique with the hybridization procedure enables the identity of the abnormal chain of a haemoglobin to be determined on 1 mg. quantities of material. The method has been verified by repeating the hybridization of the abnormal α-chain haemoglobin I with the abnormal β-chain haemoglobin C (Itano & Robinson, 1959). As in the original experiment, an extra haemoglobin zone appeared in the position of haemoglobin A. This zone comprises haemoglobin A itself, and the doubly abnormal haemoglobin I/C of net charge equal to that of A.

Dissociation and recombination of a haemoglobin G from Ibadan (Shooter, Skinner, Garlick & Barnicot, 1960) with the abnormal β-chain haemoglobin S produced two new haemoglobin species, one having the mobility of haemoglobin A and the other a mobility close to that of haemoglobin C. With haemoglobin J an additional zone appeared in the position of haemoglobin A. On the other hand, no new haemoglobin species were formed in the hybridization of haemoglobin G with I. This haemoglobin G has therefore an abnormal α-chain and the composition of the hybrids formed in the experiments with S and J are given in the following equations:

$$\underset{\text{Hb-G}}{2\alpha_2^G\beta_2^A} + \underset{\text{Hb-S}}{2\alpha_2^A\beta_2^S} \rightarrow \underset{\substack{\text{Hybrid}\\\text{Hb-G/S}}}{\alpha_2^G\beta_2^S} + \underset{\text{Hb-G}}{\alpha_2^G\beta_2^A} + \underset{\text{Hb-S}}{\alpha_2^A\beta_2^S} + \underset{\substack{\text{Hybrid}\\\text{Hb-A}}}{\alpha_2^A\beta_2^A} \quad (1)$$

$$\underset{\text{Hb-G}}{2\alpha_2^G\beta_2^A} + \underset{\text{Hb-J}}{2\alpha_2^A\beta_2^J} \rightarrow \underset{\text{Hb-G}}{\alpha_2^G\beta_2^A} + \underset{\substack{\text{Hybrid}\\\text{Hb-G/J}}}{\alpha_2^G\beta_2^J} + \underset{\substack{\text{Hybrid}\\\text{Hb-A}}}{\alpha_2^A\beta_2^A} + \underset{\text{Hb-J}}{\alpha_2^A\beta_2^J} \quad (2)$$

If no abnormal α-chain haemoglobin is available a positive identification of an abnormal β-chain haemoglobin may be obtained with haemoglobin H, which is known to be a β-chain tetramer (Jones, Schroeder, Balog & Vinograd, 1959). In this case haemoglobin A is formed when haemoglobin H is hybridized with an abnormal β-chain haemoglobin, e.g. haemoglobin E, but not when it is hybridized with an α-chain abnormal haemoglobin. Application of these techniques has now shown that haemoglobin G (Edington, Lehmann & Schneider,

1955), D_{Cyprus}, $D_{Frankfurt}$, D_{γ}, $J_{Trinidad}$, N and O have abnormal β-chains, whilst K and Norfolk have abnormal α-chains.

REFERENCES

Edington, G. M., Lehmann, H. & Schneider, R. G. (1955). *Nature, Lond.*, **175**, 850.

Itano, H. A. & Robinson, E. (1959). *Nature, Lond.*, **183**, 1799.

Itano, H. A., Singer, S. J. & Robinson, E. (1959). In *Ciba Foundation Symp., Biochemistry of Human Genetics*, p. 96. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. & A. Churchill Ltd.

Jones, R. T., Schroeder, W. A., Balog, J. E. & Vinograd, J. R. (1959). *J. Amer. chem. Soc.* **81**, 3161.

Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.

Shooter, E. M., Skinner, E. R., Garlick, J. P. & Barnicot, N. A. (1960). *Brit. J. Haematol.* **6**, 140.

Vinograd, J. R., Hutchinson, N. D. & Schroeder, W. A. (1959). *J. Amer. chem. Soc.* **81**, 3168.

THURSDAY MORNING, 30 JUNE
(ANATOMY THEATRE)

(28) Partial Purification of a Brain Phosphoprotein Attached to Subcellular Particles.

By P. J. HEALD. (*Department of Biochemistry, Institute of Psychiatry, Denmark Hill, London, S.E. 5*)

It has been shown (Heald, 1959) that brain contains two major groups of phosphoproteins, one attached to particulate matter, the other soluble in aqueous sucrose solutions. The particulate phosphoprotein, which is not contained in the mitochondria, appears to be a single entity as regards the incorporation of radioactive phosphate. It is of particular interest since the radioactivity of the phosphorus increases markedly when slices of cerebral cortex, metabolizing glucose are briefly stimulated by electrical pulses. To obtain further information regarding its nature and to provide a substrate for the study of phosphorylation and dephosphorylation mechanisms, a soluble preparation has been obtained from ox brain. The phosphoprotein is firmly bound in the particles but can be solubilized by treatment with a secondary alkyl sulphate and further purified by precipitation with acetone and sodium chloride. The preparations so far obtained are purified 14–15 times in terms of phosphorus, contain little nucleic acid, 8–8·6% N, 0·3–0·6% P, and 40% lipid, and the yield was 25%. The lipids extracted either by ethanol–ether (3:1, v/v) or chloroform–methanol (2:1, v/v) contain 0·9–1·2% P, 1–2% N, and 1·5–2·6% carbohydrate (as galactose). Cholesterol is absent. Ester analysis (Shapiro, 1953) accounts for the remainder of the lipids, which are therefore presumably neutral triglycerides. The lipid-free residues contain 11·2–11·6% N and 0·1–0·24% P, 50–75% of which is labile in alkali. Qualitative paper chromatography of acid hydrolysates revealed an amino acid pattern suggestive of an acidic protein. When incubated either with a fortified cerebral dispersion or with rat-liver mitochondria in the presence of radioactive phosphate, the preparations are phosphorylated, radioactive phosphorylserine being detected in hydrolysates of the phosphoproteins at the end of the incubation.

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REFERENCES

Heald, P. J. (1959). *Biochem. J.* **73**, 132.

Shapiro, B. (1953). *Biochem. J.* **53**, 663.

(29) Metal Linkages in the Thymus nucleohistone and their Damage by X-Irradiation.

By U. HAGEN (introduced by W. J. WHELAN). (*Heiligenberg Institut, Heiligenberg, Germany*)

Kirby (1957, 1958) suggested, that deoxyribonucleic acid (DNA) is bound to proteins by co-ordinated metal linkages besides electrovalent linkages. According to the present author, DNA may be extracted from tissues with 90% phenol only in the presence of certain anions, for instance trichloroacetate. These anions apparently break the bivalent metal linkages between DNA and protein. Recently we found (Hagen, 1960) that below a final concentration of 1% trichloroacetate, only a small yield (10–15%) of DNA can be obtained, whereas with 2% trichloroacetate all of the DNA can be extracted. This indicates the existence of a threshold concentration of trichloroacetate for complete extraction of DNA.

We observed this behaviour, characteristic for metal linkages between DNA and protein, not only at the extraction of whole tissue but also at the same treatment of thymus nucleohistone. This nucleohistone was extracted from the thymus with M-NaCl and precipitated by dilution to 0·14M-NaCl. No DNA at all can be obtained with trichloroacetate-phenol from the 'histone-free deoxyribonucleoprotein' of Monty & Dounce (1958). This means that in the living cell DNA is bound by metal linkages to histone, not to the 'residual protein' described by Mirsky & Pollister (1946).

After X-irradiation of animals (2–6 hr. after 200–1000 r.), a higher yield of DNA can be obtained at the extraction with 0·5 and 1% trichloroacetate from the thymus tissue (Hagen, 1960) as well as from isolated thymusnucleohistone. This effect

indicates a damage or a labilization of metal linkages by the X-irradiation. If calcium ions are added to the nucleohistone of irradiated animals, the raised yield of DNA decreases again to normal values. Not only calcium but, also magnesium is able to abolish this labilization effect of irradiation and to 'repair' the irradiation damage of metal linkages.

This phenomenon provides a basis to determine the amount of metal linkages in the thymus nucleohistone. At 6 hr. after 800 r. of whole-body irradiation, at a nearly complete destruction of the metal linkages, 0·55 % of Ca^{2+} per nucleohistone is necessary to repair the irradiation damage. This value is very similar to the calcium concentration in nucleohistone measured by Frick (1958) with a quite different experimental technique (0·54–0·66 % Ca^{2+} per deoxyribonucleoprotein).

REFERENCES

Frick, G. (1958). *Exp. Cell Res.* **15**, 191.
Hagen, U. (1960). *Nature, Lond.*, (in the Press).
Kirby, K. S. (1957). *Biochem. J.* **66**, 495.
Kirby, K. S. (1958). *Biochem. J.* **70**, 260.
Mirsky, A. E. & Pollister, A. W. (1946). *J. gen. Physiol.* **34**, 117.
Monty, K. J. & Dounce, A. L. (1958). *J. gen. Physiol.* **41**, 595.

(30) Low-Angle X-ray-Scattering Studies of Ribonucleic Acid in Solution.

By S. N. Timasheff* and V. Luzzati (introduced by W. J. Whelan). (*Centre de Recherches sur les Macromolécules, Strasbourg, France*)

Samples of ribonucleic acid (RNA) from three sources were studied, namely, ascites tumour cell (sample I), *Escherichia coli* (sample II) and yeast (sample III). Sample I was prepared by Colter & Brown according to their method (1956) and contained only material precipitated by M-NaCl; samples II and III were given to us by Professor J. P. Ebel; they were not fractionated. All materials were lyophilized. Ultracentrifugal examination showed sample I to have essentially two peaks with $S_{20,w}$ 18 (37 %) and 31 (63 %) and to be identical with material previously described (Colter & Brown, 1956). Sample II was highly aggregated and sample III sedimented in a broad band with an $S_{20,w}$ distribution between 4 and 15.

The low-angle scattering experiments were performed with dilute isotropic solutions (0·5–3·5 % by wt.) and the intensities were measured on the absolute scale by direct comparison with the energy of the direct beam (Luzzati, 1960). All the samples were examined in water and in a buffer at pH 6·8–7·0 having I 0·15 (0·14 M-NaCl–0·01 M-phosphate) at $3 \pm 1°$.

The results obtained in the buffer were identical for the three samples of RNA over the entire angular range covered. The interpretation of the experimental data (Luzzati, 1960) shows that over a short distance the molecules are rod-like in shape. Two parameters of the rods have been determined: the radius of gyration around the axis, R_c, and the linear specific molecular weight (μ). R_c is 8·5 Å; μ is 116 ± 5, assuming the partial specific volume, $\bar{V}$, to be 0·578 (Cohen & Stanley, 1942). Both parameters are very similar to those found in deoxyribonucleic acid (DNA) under the same experimental conditions (Luzzati, Nicolaieff & Masson, unpublished results), i.e. R_c 8·4, μ 110, and $\bar{V}$ 0·595. In one respect, however, the small-angle X-ray diffraction patterns of DNA and RNA are different. Dilute solutions of DNA behave as a set of independent rigid rods down to $s = 1/300$ Å [$s = (2 \sin \theta)/\lambda$]. On the contrary, RNA molecules are either flexible or they form aggregates, as is shown by the inner part of the diffraction pattern. When compared with light-scattering data (Timasheff, Brown, Colter & Davies, 1958; Kronman, Timasheff, Colter & Brown, 1960) obtained on similar preparations of RNA from ascites tumour cell, the X-ray observations are found to be well consistent with the presence of small swollen particles.

The effect of lowering electrolyte concentration was discussed.

REFERENCES

Cohen, A. & Stanley, A. (1942). *J. biol. Chem.* **144**, 589.
Colter, J. S. & Brown, R. A. (1956). *Science*, **124**, 1077.
Kronman, M. J., Timasheff, S. N., Colter, J. S. & Brown, R. A. (1960). *Biochim. biophys. Acta* (in the Press).
Luzzati, V. (1960). *Acta cryst., Camb.*, (in the Press).
Timasheff, S. N., Brown, R. A., Colter, J. S. & Davies, M. (1958). *Biochim. biophys. Acta*, **27**, 662.

* Senior Postdoctoral Fellow of the National Science Foundation, U.S.A.

(31) Nucleic Acid Turnover and Enzyme Synthesis in Resting Bacteria.

By M. H. Proctor*. (*Department of Biochemistry, University of Cambridge*)

Using the paper-chromatographic technique of Wyatt (1951) for the separation of free purine and pyrimidine bases, the base ratios of the nucleic acid of a gram-variable bacterium have been determined as adenine, 1·00; guanine, 1·10; cytosine, 0·65; uracil, 0·67; thymine, 0·15. Deoxyribonucleic acid

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(DNA) and ribonucleic acid (RNA) were isolated from the organism by the procedure of Dutta, Jones & Stacey (1953) and the base ratios of the isolated materials were for DNA: adenine, 1·00; guanine, 1·18; cytosine, 1·02; thymine, 0·94, and for RNA: adenine, 1·00; guanine, 1·34; cytosine, 0·88; uracil, 0·82. The resting cells used for the present experiments on enzyme induction contained by weight 9·55% of RNA and 1·9% of DNA, estimated by the method of Burton (1955).

Chromatography and radioautography indicate that [^{14}C]uracil is a source of nucleic acid uracil and cytosine; [^{14}C]adenine is a source of both adenine and guanine; and [^{14}C]guanine is incorporated mainly as guanine with a trace conversion into adenine. The rate of turnover of [2-^{14}C]uracil, [8-^{14}C]adenine, and [8-^{14}C]guanine in the nucleic acids are linear and have been estimated in resting cells to be 0·3%/hr. for adenine, 0·4%/hr. for guanine, and 0·15%/hr. for uracil. Turnover has been estimated from the rate of release of radioactivity from the fraction of the cells precipitated by cold trichloroacetic acid to that soluble in cold trichloroacetic acid. The compounds in which the counts resided were isolated and characterized by chromatographic and spectral methods. During the 4 hr. observation period transfer of radioactivity to compounds less complex than purines and pyrimidines was negligible; cell lysis and leakage were negligible.

The addition of melibiose, which is an inducer for the formation of *o*-nitrophenyl β-D-galactosidase, was followed by synthesis of the enzyme. The synthesis became linear after a lag period of 40 min. During the first 30–60 min. after the addition of inducer, the rates of turnover of the adenine, guanine, and uracil increased some 2–5 times and then resumed the linear rates reported above.

REFERENCES

Burton, K. (1955). *Biochem. J.* **61**, 473.
Dutta, S. K., Jones, A. S. & Stacey, M. (1953). *Biochim. biophys. Acta*, **10**, 613.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

(32) The Probable Alkylation of Liver Ribonucleic Acid by the Hepatic Carcinogens Dimethylnitrosamine and Ethionine.

By E. FARBER* and P. N. MAGEE. (*Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey*)

Ethionine (Farber, 1959) and dimethylnitrosamine (DMN) (Magee & Barnes, 1956) inhibit protein synthesis and induce cancer in the liver of rats. Ethionine is incorporated into liver protein apparently by peptide bonds (Levine & Tarver, 1951). Radioactivity is bound to protein and to nucleic acid of rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine (Magee & Hultin, 1958).

The intraperitoneal injection of [^{14}C]DMN or of L-[1-*ethyl*-^{14}C]ethionine into female rats caused liver ribonucleic acid (RNA) to be labelled much more than total liver protein. The ratio of the specific radioactivity of RNA to proteins with DMN was 5·1–5·5:1 and with ethionine was 2·4–2·6:1. This is in contrast to the ratios with L-[*Me*-^{14}C]methionine (0·43–0·52:1), L-[^{11}S]methionine (0·021–0·028:1), uniformly labelled L-[^{14}C]leucine (0·046:1), DL-[^{14}C]valine (0·036:1) and sodium [^{14}C]formate (0·31–0·32:1). Hydrolysis of the RNA with N-HCl to release free purines, followed by chromatography on Dowex-50 (H^+), revealed that after treatment with DMN, over 80% of the radioactivity is in a narrow band about midway between the guanine and adenine peaks. This peak of radioactivity coincides with a small peak in ultraviolet absorption which has a spectrum identical with that of an authentic sample of 7-methylguanine. Methylation after treatment with DMN *in vivo* may be analogous to the formation of 7-methylguanine by the action of dimethyl sulphate on guanylic acid *in vitro* (Lawley & Wallick, 1957). With ethionine, at least two peaks of radioactivity were found in the purine region not corresponding to guanine or adenine. It is possible that these are ethylated purines. Thus the two carcinogens DMN and ethionine appear to alkylate liver RNA to produce chemically abnormal components. This is consistent with the suggested importance of alkylation of cell constituents in carcinogenesis (Haddow, 1958) and offers some experimental evidence for the possible importance of altered RNA in the early stages of cancer production (Darlington, 1948; Todd, 1959).

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REFERENCES

Darlington, C. D. (1948). *Brit. J. Cancer*, **2**, 118.
Farber, E. (1959). *Arch. Path.* **67**, 1.
Haddow, A. (1958). *Brit. med. Bull.* **14**, 79.
Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
Levine, M. & Tarver, H. (1951). *J. biol. Chem.* **192**, 835.
Magee, P. N. & Barnes, J. M. (1956). *Brit. J. Cancer*, **10**, 114.
Magee, P. N. & Hultin, T. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 14–50.
Todd, A. (1959). *Brit. med. J.* **2**, 517.

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(33) Some Effects of Glutathione on Protamine-treated Amino Acid-Activating Systems.

By PRISCILLA HELE. (*Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London, W.12*)

Preparations of amino acid-activating systems from which soluble RNA (S-RNA) has been removed (Finch & Hele, 1959; Hele & Finch, 1960) become unstable on further purification, unless kept in 100 mM-glutathione. Schweet (personal communication) observed similar effects with guinea-pig-liver preparations low in S-RNA; 'pH 5·0 fractions' (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958), rich in S-RNA, are stable without glutathione.

This difference led to the further study of 'pH 5·0 fractions' and soluble-protein preparations, low in S-RNA, both from rat liver (Hele & Finch, 1960). These 'RNA-low' fractions were further purified by adsorption on to calcium triphosphate gel, and elution with 10 mM-sodium phosphate (pH 8·0)–saturated $(NH_4)_2SO_4$ (3:1, v/v). Precipitation of the eluted protein with recrystallized $(NH_4)_2SO_4$ (at 40% saturation) gave an 'RNA-low fraction', which catalysed pyrophosphate exchange with a number of amino acids, that with leucine and lysine being of most interest. The exchange rate with both these amino acids, with 15 mM-$MgCl_2$, was about 200·0 μm-moles/min./mg. of protein, more than 10 times the rate without added amino acid. Half-maximal velocity occurred with 0·1 mM-lysine or leucine.

The stability of pyrophosphate-exchange activity was studied by pre-incubating enzyme preparations before assay for varying times at 37°, without substrates, and in the presence of various combinations of $(NH_4)_2SO_4$ (probably a source of heavy metal ions), 2:6-dichlorophenolindophenol, ethylenediaminetetra-acetic acid (EDTA), or glutathione. Under conditions where 'RNA-low fractions', or ribonuclease-treated 'pH 5 fractions' lost 60–90% of their activity, the 'pH 5·0 fractions' lost 10%. Complete protection was afforded by glutathione or EDTA, but none by added yeast RNA or rat-liver S-RNA.

Transfer of [^{14}C]leucine and [^{14}C]lysine to added S-RNA (2–4 μm-moles/mg. of S-RNA with either amino acid) was catalysed by the 'RNA-low fraction', this transfer being affected by glutathione, EDTA and various pre-incubation treatments.

These observations raise the possibility that, in the 'pH 5·0 fraction', the terminal nucleotide sequence of the S-RNA (Hecht, Stephenson & Zamecnik, 1959) confers protection upon a thiol group(s), perhaps required for the transfer of amino acids from amino-acyl adenylates to S-RNA.

The effects of sulphydryl compounds or 2:6-dichlorophenolindophenol observed in systems using RNA-low 'S-protein' (Sachs, 1957; Rendi & Campbell, 1959; Rendi & Hultin, 1959*a*, *b*, 1960) may be related to the phenomena described here.

REFERENCES

Finch, L. R & Hele, P. (1959). *Biochem. J.* **73**, 11P.
Hecht, L. I., Stephenson, M. L. & Zamecnik, P. C. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 505.
Hele, P. & Finch, L. R. (1960). *Biochem. J.* **75**, 352.
Hoagland, M. B., Stephenson, M. L., Scott, F. J., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Rendi, R. & Campbell, P. N. (1959). *Biochem. J.* **72**, 435.
Rendi, R. & Hultin, T. (1959*a*). *Exp. Cell Res.* **17**, 540.
Rendi, R. & Hultin, T. (1959*b*). *Exp. Cell Res.* **18**, 542.
Rendi, R. & Hultin, T. (1960). *Exp. Cell Res.* **19**, 253.
Sachs, H. (1957). *J. biol. Chem.* **228**, 23.

(34) Incorporation of Radioactive Amino Acids into Serum Albumin by Isolated Rat-liver Ribosomes.

By A. KORNER. (*Department of Biochemistry, University of Cambridge*)

Ribosomes can be isolated from rat liver in a state in which they incorporate radioactive amino acids into protein for some hours (Korner, 1959*a*, 1960). This communication reports the incorporation of labelled amino acids into serum albumin in this system.

Ribosomes, prepared and washed free of deoxycholate and suspended in a buffered salt medium were incubated with radioactive amino acids as previously described (Korner, 1960). Samples of the incubation mixture were withdrawn at various times and the reaction stopped by addition of ice-cold 0·44M-sucrose containing excess of unlabelled amino acids and the ribosomes separated from the soluble fraction by centrifuging at 105000 *g* for 1 hr. Rat albumin (10 mg.) was added to each fraction to act as a carrier.

The ribosomes were treated with ribonuclease at 37° for 30 min. or in other ways to release protein from the ribosomes. The protein released from the ribosomes and that of the soluble fraction, were separately precipitated with 10% trichloroacetic acid and the precipitate extracted with ethanol. This procedure renders soluble all the serum albumin together with some other proteins (Korner & Debro, 1956). Ethanol and trichloroacetic acid were removed by dialysis and the proteins were treated with rabbit serum containing antibody to ox-serum albumin to remove non-specific precipitable protein. Rabbit serum containing antibody to rat-serum albumin was then added, the precipitated antibody–antigen complex spun off and the unprecipitated protein added to the 'non-albumin' protein which had not dissolved in ethanol after precipitation with trichloroacetic acid. It is possible to

separate albumin from its antibody–antigen complex by treatment with ethanol–trichloroacetic acid (Peters, 1958) and then to submit the albumin to procedures designed to demonstrate its identity.

The albumin from the soluble fraction and from the ribosomes, and the non-albumin protein from these two sources, was prepared for counting, and its radioactivity estimated as previously described (Korner, 1959*b*). Both albumin fractions were steadily and progressively labelled during incubation for about 3 hr. Their specific activities were of the same order as those of the non-albumin fractions even though they had been greatly diluted by carrier albumin. Very low levels of amino acid incorporation into albumin were obtained when the energy source was omitted from the incubation medium.

REFERENCES

Korner, A. (1959*a*). *Biochim. biophys. Acta*, **35**, 554.
Korner, A. (1959*b*). *Biochem. J.* **73**, 61.
Korner, A. (1960). *Biochem. J.* (in the Press).
Korner, A. & Debro, J. R. (1956). *Nature, Lond.*, **178**, 1067.
Peters, T., jun. (1958). *J. Amer. chem. Soc.* **80**, 2700.

(35) The Pyridine Nucleotide Content of Mitochondria of Ehrlich Ascites Tumour Cells.

By P. Borst and J. P. Colpa-Boonstra (introduced by E. C. Slater). (*Laboratory of Physiological Chemistry, University of Amsterdam, The Netherlands*)

The isolation procedure for ascites-cell mitochondria described by Borst (1960) has been improved to yield a routine preparation of constant quality containing 15–25 mg. of protein. These mitochondria show adenosine diphosphate respiratory-control indices of 4·1–5·7 with glutamate, a latent adenosine triphosphatase, normal P:O ratios with succinate and diphosphopyridine nucleotide (DPN)-linked substrates and a low ratio (0·25) with ascorbate + cytochrome *c* (P. Borst, unpublished results).

With this mitochondrial preparation we have reinvestigated the DPN requirement of DPN-linked oxidations, reported for tumour mitochondria in general (Weinhouse, 1955) and recently confirmed for ascites-cell mitochondria (Hawtrey & Silk, 1960). Addition of mM-DPN to ascites-cell mitochondria oxidizing 0·02 M-α-oxoglutarate + 0·02 M-malonate, 0·02 M-glutamate or 0·02 M-pyruvate + 2 mM-malate had no significant effect on the uptake of oxygen.

To verify these unexpected results pyridine nucleotides were determined by fluorimetric methods, applied to acid and alkali extracts of fresh mitochondrial preparations, using separate enzymic analyses for DPN, triphosphopyridine nucleotide (TPN), DPNH and DPNH + TPNH (Ciotti & Kaplan, 1957). The average values (range in brackets), expressed as μmoles/g. of mitochondrial protein of 4–5 preparations, were: DPNH, 2·1 (1·5–2·8); DPN, 3·2 (1·5–4·4); TPNH, 0·5 (−0·3–1·0); TPN, 0·0 (−0·1–+0·3). Since the TPNH content was determined by difference, the presence of TPNH in ascites-cell mitochondria cannot yet be considered as established. However, the DPNH + DPN content is clearly comparatively high. A recent average value obtained in this laboratory by the same procedure for the total DPN content of 10 preparations of fresh rat-liver mitochondria was 3·5 μmoles/g. of protein (2·1–4·9), while the values obtained fluorimetrically by other workers (Jacobson & Kaplan, 1957; Purvis, 1960) are even lower.

From these results it seems probable that the DPN requirement found for ascites-cell mitochondria by Hawtrey & Silk (1960) is an artifact of their isolation procedure. Moreover, the finding that the mitochondria of a malignant and biochemically 'typical' tumour (Warburg, 1956) contain what could well be called an abundance of DPN, make it improbable that the proposed DPN deficiency of tumour mitochondria (Weinhouse, 1955) can be the cause of the biochemical characteristics of tumour metabolism.

This work was supported in part by a grant from the Netherlands Organization for National Health Research, T.N.O., and the Koningin Wilhelmina Fund for Cancer Research.

REFERENCES

Borst, P. (1960). *J. biophys. biochem. Cytol.* (in the Press).
Ciotti, M. M. & Kaplan, N. O. (1957). In *Methods in Enzymology*, vol. **3**, p. 890. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hawtrey, A. O. & Silk, M. H. (1960). *Biochem. J.* **74**, 21.
Jacobson, K. B. & Kaplan, N. O. (1957). *J. biol. Chem.* **226**, 603.
Purvis, J. L. (1960). *Biochim. biophys. Acta*, **38**, 435.
Warburg, O. (1956). *Science*, **123**, 309.
Weinhouse, S. (1955). *Advanc Cancer Res.* **3**, 269.

Thursday Morning, 30 June
(Physiology Theatre)

(36) The Extraction and Measurement of Urinary 17-Oxo steroid Hydrogen Sulphates.

By Jean McKenna and J. K. Norymberski. (*Medical Research Council Unit for Research on the Chemical Pathology of Steroids, Jessop Hospital for Women, Sheffield*)

Routine determinations of urinary steroid hydrogen sulphates are based on the preferential cleavage of their sulphuric ester bond either by enzymes, or by heating at or about pH 7, or by acid-catalysed solvolysis (Roy, 1956; Burstein & Lieberman, 1958;

Fotherby, 1959; and references there cited). Solvolytic cleavage in the absence of acid (Cohen & Oneson, 1953; McKenna & Norymberski, 1957) is a method of choice since it proceeds under mild conditions, specifically, and regardless of the position or orientation of the ester group, but cannot be applied to crude extracts of urine (Cohen & Oneson, 1953). Recently, a method was reported for the direct chromatographic estimation of the hydrogen sulphates of dehydro*epi*androsterone, androsterone, and aetiocholanolone in an extract obtained with ethyl acetate from urine half-saturated with ammonium sulphate (Bush & Gale, 1960).

The partition coefficients of steroid hydrogen sulphates and of their salts for water–chloroform strongly favour the aqueous phase. We find that in the presence of the pyridinium ion chloroform is the favoured phase. Aqueous solutions of the salts (sodium, potassium, or pyridinium) of dehydro*epi*androsterone sulphate and of related substances were made 0·3M in pyridinium sulphate and extracted with chloroform (0·5–1 vol.); the extracts contained 80–100% of the sulphates in the form of their pyridinium salts. Under identical conditions, extraction of urine gave similar recoveries of added material.

The urinary extracts were found pure enough to be submitted to solvolysis by dioxan without addition of acid, following which the 17-oxo steroids derived from their sulphates were measured as a group by means of the Zimmermann reaction or resolved on paper in Bush's system *A* (Bush, 1952; Brooks, 1958) into their individual components. Further concentration of the urinary steroid sulphates was brought about by extracting their chloroform solutions with water (0·5 vol.) and the aqueous solutions, after addition of pyridinium sulphate, again with chloroform.

REFERENCES

Brooks, R. V. (1958). *Biochem. J.* **68**, 50.
Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Bush, I. E. & Gale, M. M. (1960). *Biochem. J.* (in the Press).
Cohen, S L. & Oneson, I. B. (1953). *J. biol. Chem.* **204**, 245.
Fotherby, K. (1959). *Biochem. J.* **73**, 339.
McKenna, J. & Norymberski, J. K. (1957). *J. chem. Soc.* p. 3889.
Roy, A. B. (1956). *Biochem. J.* **62**, 41.

(37) A Urinary Conjugate of Indolyl-3-carboxylic Acid.

By S. Balakrishnan and R. Rodnight. (*Department of Biochemistry, Institute of Psychiatry, London, S.E.* 5)

In the course of studying urines from mentally ill subjects for acidic indoles by paper chromatography an unknown compound reacting with Ehrlich's reagent was observed in extracts prepared by running urine (at pH 7) through columns of De-Acidite *E* (Permutit Co. Ltd.) in the OH^- form, washing the resin and eluting the absorbed acids with methanolic ammonia. The spot gave a purple colour within 1 min. of dipping the chromatogram in Ehrlich's reagent (Smith, 1958), which faded to blue-grey within 3 hr. The R_F values were: propanol–ammonia–water (80:5:15), 0·33; butanol–acetic acid–water (60:15:25), 0·53.

Some 15 mg. of the substance was obtained free of other indoles by chromatography of about 1 l. of urine from a high excretor on De-Acidite *E* and paper. By hydrolysis with saturated $Ba(OH)_2$ (1 hr., 100°) followed by acidification and extraction with ethyl acetate, an acidic indole was isolated with identical R_F values (7 solvents) and properties with respect to reaction with Ehrlich's reagent (pink colour after 24 hr.), to synthetic indolyl-3-carboxylic acid (Doyle, Ferrier, Holland, Mehta & Naylor, 1956). By acid hydrolysis a compound was obtained with the chromatographic properties and reactions of glucuronic acid, thus making the probable identity of the parent substance indolyl-3-carboxylic acid glucuronide. This was confirmed by incubation with β-glucuronidase for 24 hr. at pH 4·5. The conjugate was excreted in detectable quantities by 11 out of 35 normal subjects (31%) examined (range: 20–60 mg./24 hr.) and by 35 out of 55 mental hospital patients (64%) (range: 20–120 mg./24 hr.).

Free indolyl-3-carboxylic acid has been reported in urine (Armstrong, Shaw, Gortatowski & Singer, 1958) and probably originates from the action of the gut flora on tryptophan. When indolyl-3-carboxylic acid was fed to a normal subject some 10% was excreted over 12 hr. as the free acid, but no increase in the glucuronide was observed. Instead, an as yet unidentified compound was excreted with the same R_F values as the glucuronide and which gave a pink colour with Ehrlich's reagent only after storing the paper for 24 hr. in an atmosphere of HCl vapour.

We are grateful to the authorities of the British Council (Colombo Plan Fellowships' Section) for the award of a Fellowship to one of us (S. Balakrishnan) and to the Board of Governors of the Bethlem Royal Hospital and the Maudsley Hospital for financial support.

REFERENCES

Armstrong, M. D., Shaw, K. N. F., Gortatowski, M. J. & Singer, H. (1958). *J. biol. Chem.* **232**, 17.
Doyle, F. P., Ferrier, W., Holland, D. O., Mehta, M. D. & Naylor, J. H. C. (1956). *J. chem. Soc.* p. 2853.
Smith, I. (1958). *Chromatographic Techniques.* London: W. Heinemann Ltd.

(38) Formation of *N*-Acetyl-*S*-ethyl-L-cysteine *S*-oxide from Bromoethane in the Animal Body.

By A. E. R. THOMSON and L. YOUNG. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

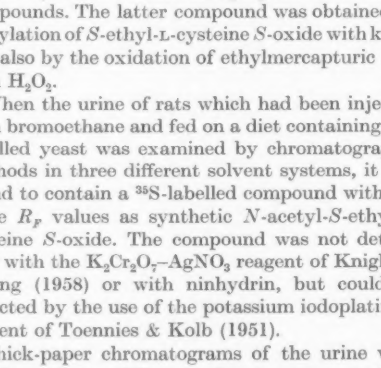
Following the administration of bromoethane to the rat, the excretion of two sulphur-containing metabolites of this compound was observed, one of which was identified as ethylmercapturic acid, i.e. *N*-acetyl-*S*-ethyl-L-cysteine (Thomson, Maw & Young, 1958). The other compound was not identified, but evidence has now been obtained that it is the sulphoxide of ethylmercapturic acid, i.e. *N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide:

$$C_2H_5Br \rightarrow C_2H_5{\cdot}SO{\cdot}CH_2{\cdot}CH(NH{\cdot}CO{\cdot}CH_3){\cdot}CO_2H$$

S-Ethyl-L-cysteine and *S*-ethyl-L-cysteine *S*-oxide, *N*-acetyl-*S*-ethyl-L-cysteine and *N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide were prepared as reference compounds. The latter compound was obtained by acetylation of *S*-ethyl-L-cysteine *S*-oxide with keten and also by the oxidation of ethylmercapturic acid with H_2O_2.

When the urine of rats which had been injected with bromoethane and fed on a diet containing ^{35}S-labelled yeast was examined by chromatographic methods in three different solvent systems, it was found to contain a ^{35}S-labelled compound with the same R_F values as synthetic *N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide. The compound was not detectable with the $K_2Cr_2O_7$–$AgNO_3$ reagent of Knight & Young (1958) or with ninhydrin, but could be detected by the use of the potassium iodoplatinate reagent of Toennies & Kolb (1951).

Thick-paper chromatograms of the urine were developed in two different solvent systems, and the radioactive metabolite was located and eluted with water. The eluates were then examined by radiochromatography after various treatments. Only ethylmercapturic acid was detected after reduction with thioglycollic acid. After an eluate had been incubated with a preparation of pig-kidney deacetylase it contained *S*-ethyl-L-cysteine *S*-oxide and when it was heated in 6N-HCl there was formation of *S*-ethyl-L-cysteine.



By means of paper chromatography it was shown that synthetic *N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide behaved in the same manner when subjected to these processes. Furthermore, ethylmercapturic acid and *S*-ethyl-L-cysteine were isolated, the former after reducing the synthetic mercapturic acid sulphoxide with thioglycollic acid and the latter after decomposing it with 6N-HCl.

It is believed that the present work provides the first evidence for the formation of a mercapturic acid sulphoxide in the animal body.

REFERENCES

Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Thomson, A. E. R., Maw, G. A. & Young, L. (1958). *Biochem. J.* **69**, 23P.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.

(39) Isolation of Urinary Steroid Glucuronosides.

By FRANCES FOGGITT and A. E. KELLIE. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

Standard methods for the analysis of urinary steroids are indirect, involving preliminary hydrolysis of the glucuronoside and sulphate conjugates. Procedures are, however, available for the quantitative extraction of steroid conjugates (Edwards, Kellie & Wade, 1953; Bush & Gale, 1957) and recently methods have been described for the separation of steroid glucuronosides from steroid sulphates (Crepy, Jayle & Meslin, 1957; Pelzer & Staib, 1957; Barlow & Kellie, 1959) and individual steroid conjugates from each other (Lewbart & Schneider, 1955, 1956; Bush, 1957). The present paper deals with the application of these methods for the isolation and identification of 17-oxo steroid glucuronosides and of corticosteroid glucuronosides from human urine.

Urines obtained from normal subjects following the administration of corticotropin were adjusted to pH 2 and extracted by the method of Edwards *et al.* (1953). The glucuronoside fraction obtained by separation on an alumina column was subsequently analysed by partition column chromatography on Celite 535 using systems related to those described by Bush (1957). Separation of the following components of the 17-oxo steroid glucuronoside fraction was achieved; androsterone glucuronoside, aetiocholanolone glucuronoside, 11-oxo-aetiocholanolone glucuronoside, 11β-hydroxyandrosterone glucuronoside and 11β-hydroxyaetiocholanolone glucuronoside. Using similar columns with more polar partition systems the glucuronosides of the following corticosteroids, 3α:5β-tetrahydro S (THS),

3α:5α-tetrahydro B, 3α:5β-tetrahydro E (THE) and 3α:5β-tetrahydro F (THF) were separated and identified. In no case have these isolated steroid glucuronosides been obtained, as such, in crystalline form and evidence of identity is based, in part, upon preliminary hydrolysis of the purified steroid glucuronoside using β-glucuronidase followed by chromatographic and infrared identification of the free steroid.

In the case of androsterone, aetiocholanolone, THS, THE and THF glucuronosides sufficient material was available to obtain the steroid glucuronosides in crystalline form as the triacetate methyl esters. After treatment of the steroid glucuronoside with diazomethane and acetylation with acetic anhydride and pyridine the product was purified by partition chromatography on Celite. The preparation of synthetic compounds for comparison is underway at present. In the course of this study the infrared absorption spectra of a large number of steroid glucuronosides and steroid glucuronoside triacetate methyl esters have been examined in solution and as KBr discs; while some differentiation is possible in the carbonyl region of the spectra, the 'fingerprint' region of all the compounds examined was similar and quite unsatisfactory as a means of identification.

REFERENCES

Barlow, J. J. & Kellie, A. E. (1959). *Biochem. J.* **71**, 86.
Bush, I. E. (1957). *Biochem. J.* **67**, 23P.
Bush, I. E. & Gale, M. (1957). *Biochem. J.* **67**, 29P.
Crepy, O., Jayle, M. F. & Meslin, F. (1957). *Acta Endocrinology*, **24**, 233.
Edwards, R. W. H., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.*, **2**, 53.
Lewbart, M. L. & Schneider, J. J. (1955). *Nature, Lond.*, **176**, 1175.
Lewbart, M. L. & Schneider, J. J. (1956). *Fed. Proc.* **15**, 300.
Pelzer, H. & Staib, W. (1957). *Clin. chim. Acta*, **2**, 407.

(40) Metabolism of 2[^{35}S]-thiohydantoin and 5-carboxymethyl-2[^{35}S]-thiohydantoin.

By G. D. Broadhead and R. Kilpatrick (introduced by J. D. Acland). (*Department of Pharmacology and Therapeutics, University of Sheffield*)

Kilpatrick, Elmore & Wood (1958) observed that the antithyroid activity of a series of thiohydantoins containing non-polar groups in the 5-position was similar to that of 2-thiouracil, while compounds containing polar groups in this position were much less active. Astwood (1945) found that 2-thiouracil derivatives having a non-polar substituent in the 6-position had greater antithyroid activities than derivatives with a polar group in this position.

Accordingly, 2[^{35}S]-thiohydantoin and 5-carboxymethyl-2[^{35}S]-thiohydantoin have been prepared and shown to be at least 98% pure by chromatography. Male albino rats were injected intraperitoneally with either 2[^{35}S]-thiohydantoin (70 μmoles/kg.) or 5-carboxymethyl-2[^{35}S]-thiohydantoin (130 μmoles/kg.). Since these compounds are unstable, urine was collected in vessels from which light was excluded and at intervals during the following 24 hr. groups of three rats were killed and samples of blood, bones, liver and thyroid taken.

Following the administration of both compounds the ^{35}S content of the serum, red-blood cells, liver and bones decreased after an initial rise during the first hour. The total ^{35}S content of the thyroids of rats which had received 2[^{35}S]-thiohydantoin increased and after 24 hr. 1 g. of thyroid contained 7·6% of the dose of radioactivity injected. The ^{35}S content of the serum after 1 hr. was 8·7% of the dose/ml. When the thyroids were homogenized with water and chromatographed in two dimensions using butanol saturated with water and ethanol–M-ammonium acetate, pH 7·5 (7·5:3, v/v) the only radioactive material which could be detected was sulphate. After 24 hr. 65% of the injected ^{35}S had been excreted in the urine and chromatography showed that 70% of the ^{35}S appeared as the parent compound, while the rest of the activity was made up of sulphate and two unidentified compounds, neither of which was 3-acetyl-2-thiohydantoin.

After the administration of 5-carboxymethyl-2-[^{35}S]-thiohydantoin no appreciable concentration of ^{35}S was found at any time in the thyroid and in contrast to 2[^{35}S]-thiohydantoin, 99% of the ^{35}S from the polar compound was excreted in the urine in 24 hr.

These results suggest that thiohydantoins containing non-polar substituents are metabolized to sulphate in the thyroid gland and that their antithyroid action is dependent on this oxidation. An increasing concentration of sulphate in the thyroid was found by Maloof & Soodak (1957) to occur after [^{35}S]thiourea, but not after 2[^{35}S]-thiouracil.

REFERENCES

Astwood, E. B. (1945). *Harvey Lect.* **40**, 195.
Kilpatrick, R., Elmore, D. T. & Wood, D. R. (1958). *Brit. J. Pharmacol.* **13**, 350.
Maloof, F. & Soodak, M. (1957). *Endocrinology*, **61**, 555.

(41) The Urea Metabolism of the Heart.

By F. Menne (introduced by P. N. Campbell). (*Institut für Physiologischie Chemie, Münster, Germany*)

The nitrogen of urea can be utilized for the synthesis of non-essential amino acids (Rose & Dekker, 1956), and the striated muscle of frogs can synthesize urea (Nekhorocheff, 1952). The present

investigation is concerned with the metabolism of urea in isolated hearts of adult rats.

(1) The hearts, washed in ice-cold Ringer solution, contained on average 203 ± 21 μg. of urea/g. wet wt. in the homogenate. [Urea was estimated by the Folin method, using urease 'Type II' (Sigma Chemical Co.).] This value, the mean of 15 experiments, is taken as the 'standard' in the following experiments.

(2) Having perfused 14 hearts for 30 min. with Krebs–Ringer buffer (38°, O_2+CO_2) by the method of Bleehen & Fisher (1954), the perfusate contained on average 175 ± 33 μg., the homogenate 61 ± 31 μg., a total of 236 ± 40 μg. of urea/g. wet wt. The urea content after perfusion was therefore 33 μg. higher than the 'standard'.

(3) Eighteen hearts perfused with 40 mg./100 ml. of urea in Krebs–Ringer buffer (30 min., 38°, O_2+CO_2) absorbed on average 249 μg. of urea/g. wet wt. If this urea had simply accumulated in the heart, they must have contained $203+249=452$ μg. of urea/g. wet wt. But in fact they had on average only 168 μg. of urea. The hearts must therefore have metabolized 284 μg. of urea, but ammonia was not detected in the perfusate or in the homogenate.

(4) Other hearts were perfused three times successively with equal amounts of Krebs–Ringer buffer (10, 20 and 30 min., 38°, O_2+CO_2). In the three perfusates together there were 166 μg. and in the homogenate, 49 μg. of urea/g. wet wt. Each heart therefore contains 263 μg. of urea, that is 12 μg. more than in the 'standard' (203 μg.). After the hearts had been washed out three times, the homogenate contained nearly the same quantity of urea (80 μg.) as the homogenate in Expt. 2 (89 μg.). This points to a fixation of urea in the heart not in agreement with the fall of concentration.

(5) Finally eight hearts were perfused with 4% NH_4Cl in Krebs–Ringer buffer three times consecutively (30 min., 38°, O_2+CO_2). They absorbed 105 μg. of NH_3/g. wet wt., but in the homogenate there were found only 24 μg. of NH_3. So on average 369 μg. of urea were given off into the perfusate (cf. 175 ± 33 μg. in Expt. 2). The concentration of urea in the heart was reduced to 48 μg. (cf. 61 ± 31 μg. in Expt. 2) so that the total content was 417 μg. This is 181 μg. higher than the corresponding value in Expt. 2 and 214 μg. higher than in the 'standard'. Evidently the hearts form urea when they take up NH_4Cl.

REFERENCES

Bleehen, N. M. & Fisher, R. B. (1954). *J. Physiol.* **123**, 260.
Nekhorocheff, J. (1952). *Arch. Sci. Physiol.* **6**, 285.
Rose, W. C. & Dekker, E. E. (1956). *J. biol. Chem.* **223**, 107.

(42) Ionic Movements with Electrical Pulses in Mammalian Cerebral Cortical Tissue.

By J. T. CUMMINS and H. MCILWAIN. [*Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E.* 5]

The effects of electrical pulses on ion movements in cerebral tissues have been examined to determine whether they are similar to those previously shown to result from stimulation of other electrically excitable systems (Hodgkin, 1958; Keynes, 1951). The experimental conditions used were similar to those reported by McIlwain (1951) and Heald & McIlwain (1956).

On incubation in oxygenated glucose salines, the tissue content of potassium salts rose to 61 ± 6 (8) μ-moles/g. and was stable at this value between 30 and 40 min. of incubation, findings similar to those of Terner, Eggleston & Krebs (1950). Application of electrical pulses of the tissue was then accompanied by a loss of potassium, of about 3% per min. during the first 3–5 min. A greater loss was observed in the absence of glucose, or when glutamate was present with or without glucose. The total sodium in the tissue did not increase to an extent equivalent to the loss of potassium. However, after application of pulses for 10 min., intracellular sodium (computed by subtracting values for the tissue chloride from those for the sodium) had increased.

Radioactive potassium was also used to measure efflux from cerebral tissue. The tissues were preincubated in glucose–bicarbonate media containing ^{42}KCl and then transferred to fresh media, where the efflux, with and without condenser pulses, was determined. Initial rates of loss of ^{42}K from the slices were approximately 4–5% of the potassium content/min. (Krebs, Eggleston & Terner, 1951) and these were increased to 6–8% by the applied pulses. The results imply an additional potassium efflux of about 250 $\mu\mu$moles of K^+/g. of tissue/applied pulse. In the absence of substrate or with glutamate, similar results were obtained, indicating that the initial loss of potassium was not substrate-dependent. It will also be noted that the time-course of the change in potassium salts shows it to continue after the major changes in the level of phosphocreatine and respiration, for after application of pulses for 10–60 sec. they have reached new, stable levels (McIlwain, 1959).

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REFERENCES

Heald, P. J. & McIlwain, H. (1956). *Biochem. J.* **63**, 231.
Hodgkin, A. L. (1958). *Proc. Roy. Soc.* B, **148**, 1.
Keynes, R. D. (1951). *J. Physiol.* **114**, 119.
Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.
McIlwain, H. (1951). *Biochem. J.* **49**, 382.
McIlwain, H. (1959). *Biochemistry and the Central Nervous System*. London: J. & A. Churchill Ltd.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.

(43) Migration of Histones from Nuclei of Isolated Cerebral Tissues kept in Cold Media.

By L. S. Wolfe and H. McIlwain. (*Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

Slices of guinea-pig cerebral cortex kept in buffered glucose media at 0° lose their normal respiratory response to applied electrical pulses. Basic proteins such as histones from calf-thymus nuclei, protamines, and extracts from cerebral cortex nuclei containing basic proteins also inhibit the isolated tissue's response to electrical stimulation (Marks & McIlwain, 1959; McIlwain, 1959). The aim of the present study was to investigate the possibility that the loss of response in cerebral slices kept in cold media was due to a reaction of the histones of the tissue with acidic sites or carriers that are essential to the metabolic response to electrical pulses.

Nuclei were isolated from unsliced and sliced guinea-pig cerebral cortex by homogenizing in 0·32 M-sucrose with 3 mM-$CaCl_2$ in an Emanuel–Chaikoff hydraulic tissue homogenizer, sedimenting in a centrifugal field of 8000 *g*-min., and washing the nuclei in 0·14 M-NaCl. Histones were extracted from the isolated nuclei with 0·2 N-H_2SO_4 followed by fractional precipitation with ethanol at $-5°$ or by extraction with 1·5 M-NaCl followed by acid extraction of the deoxyribonucleoprotein gel.

The arginine-rich histones from cerebral cortex nuclei contained 10–11% arginine and nine components were found on starch-gel electrophoresis. Basic proteins from microsomal fractions contained 4–6% arginine. Nuclear histones were lost from nuclear fractions made from sliced cerebral cortex which had been kept in cold, buffered phosphate–glucose media. Arginine-rich proteins could then be extracted from other subcellular particles of the tissue. No loss of histones occurred in nuclei from slices not subjected to cold media. Cerebral microsomal fractions were found to bind histones extracted from cerebral cortex; they also bind calf-thymus histone. It is suggested that the migration of nuclear histone in slices kept in cold media, and its combination with acidic microsomal constituents is involved in the concomitant loss of the response of the tissue to electrical stimulation. The use of basic proteins to aid in the characterization of the acidic components of the fragmented ergastoplasmic elements in the microsomal fraction was discussed.

REFERENCES

Marks, N. & McIlwain, H. (1959). *Biochem. J.* **73**, 401.
McIlwain, H. (1959). *Biochem. J.* **73**, 514.

(44) Adenosine Triphosphate and 5-Hydroxytryptamine in Cytoplasmic Particles of Small Intestine.

By W. H. Prusoff (introduced by H. Blaschko). (*Department of Pharmacology, University of Oxford*)

Some of the sites of storage for the pharmacologically-active amines are known to be rich in adenosine triphosphate (ATP); it is believed that the ATP is involved in the retention of these amines.

It has been shown that in the mucous membrane of the dog's small intestine much of the 5-hydroxytryptamine (5-HT) is present in a particulate fraction which can be sedimented by centrifugation of a cell-free homogenate in iso-osmotic (0·3 M) sucrose. This fraction has been further purified by centrifugation over a sucrose density gradient (Baker, 1958, 1959).

The fraction rich in 5-HT has now been found to contain a high concentration of ATP, as determined by the firefly luminescence method; in four experiments the molar ratio, 5-HT:ATP, was $3·1 \pm 0·6$. This fraction was subjected to a second centrifugation over a sucrose density gradient. A particulate layer, which came to an equilibrium in approximately 1·8 M-sucrose, contained the bulk of the 5-HT and ATP. In two experiments the molar ratio in this layer was found to be 2·4 and 3·0, respectively.

The experimental evidence shows that 5-HT and ATP are associated with a granular fraction and that the relative composition of these granules is retained upon repeated passage through a density gradient. It is of interest that the ratio, amine:ATP, is similar to that found in the chromaffin granules of the adrenal medulla.

REFERENCES

Baker, R. V. (1958). *J. Physiol.* **142**, 563.
Baker, R. V. (1959). *J. Physiol.* **145**, 473.

THURSDAY AFTERNOON, 30 JUNE
(ANATOMY THEATRE)

(45) Deoxyribonucleases as Growth Substances.

By R. K. ZAHN. (*Institut für Vegetative Physiologie der Universität, Frankfurt A.M., Germany*)

Brody & Thorell (1957) and Brody (1958) found the activities of deoxyribonucleases to be augmented in liver and in bone-marrow regeneration. In phage infection a rise in deoxyribonuclease activities in the host is one of the responses to injection of phage deoxyribonucleic acid (DNA) (Kunkee & Pardee, 1956). Thus it appears that a rise in deoxyribonuclease activity is closely related to propagation of DNA. If this were true then addition of deoxyribonucleases should cause a rise in mitotic rate of a given cell population. The concentration of deoxyribonucleases to be added should be such that, after the penetration barriers have been overcome, the intracellular activities will be in the same range as in regenerating tissue cells. With commercial deoxyribonuclease I (Worthington Biochemical Corporation) the optimum concentration has been found to be about 10^{-6} g./ml. Copulae of sea urchins, of turbellaria and of frogs, micro-organisms such as yeast, *Micrococcus*, *Euglena gracilis* and *Glaucoma piriformis*, and human tissue culture cells show an increased rate of cellular division when exposed to a concentration of deoxyribonuclease I of 10^{-8} to 10^{-5} g./ml. (Zahn, Müller, Müller & Zahn, 1959; Zahn, Rajewsky, Kiefer, Eitel & Zahn, 1959; Zahn & Kleinschmidt, 1959; Zahn, 1959). The question of whether these biological effects are confined to this pancreas deoxyribonuclease has been tested with other deoxyribonucleases such as a deoxyribonuclease I of *Streptococcus* (Streptodornase) (Laskowski, 1959; Potter & Laskowski, 1959; De Garilhe & Laskowski, 1955), and deoxyribonuclease II of *Micrococcus pyogenes* var. *aureus* (Catlin) (Cunningham, Catlin & De Garilhe, 1956; De Garilhe, Cunningham, Laurila & Laskowski, 1957; Cunningham, 1958, 1959; Catlin & Cunningham, 1958) and of pig spleen (Fredericq & Oth, 1958). These deoxyribonucleases too had the same effect as deoxyribonuclease I but the pig-spleen deoxyribonuclease II proved to be at least ten million times as active as all the others.

The biological actions of these enzymes are not effected by indirect extracellular hydrolytic processes on the DNA of dead cells, since addition of DNA either of a foreign or of the same species will prevent the biological effects of the enzymes completely, whereas addition of deoxyribonucleotides does not affect their activity at all. It is therefore assumed that the deoxyribonucleases, or essential parts thereof, are able to penetrate into the cells. The fact that addition of surface-active agents greatly enhances the effects of deoxyribonuclease points in this direction. In the cell, the site of deoxyribonuclease action must be closely related to the mitotic mechanisms, since the mitogenic hormone oestradiol-17β strongly enhances the effect of deoxyribonuclease. This is a highly specific action since oestradiol-17α does not provoke this response. In the light of this experimental evidence deoxyribonucleases seem to exhibit all the features of a growth substance.

REFERENCES

Brody, S. (1958). *Nature, Lond.*, **182**, 1386.
Brody, S. & Thorell, B. (1957). *Biochim. biophys. Acta*, **25**, 579.
Catlin, B. W. & Cunningham, L. (1958). *J. gen. Microbiol.* **19**, 522.
Cunningham, L. (1958). *J. Amer. chem. Soc.* **80**, 2546.
Cunningham, L. (1959). *Ann. N.Y. Acad. Sci.* **81**, 788.
Cunningham, L. B., Catlin, B. C. & De Garilhe, M. P. (1956). *J. Amer. chem. Soc.* **78**, 4642.
De Garilhe, M. P., Cunningham, L., Laurila, U. R. & Laskowski, M. (1957). *J. biol. Chem.* **224**, 751.
De Garilhe, M. P. & Laskowski, M. (1955). *J. biol. Chem.* **215**, 269.
Fredericq, E. & Oth, A. (1958). *Biochim. biophys. Acta*, **29**, 281.
Kunkee, R. E. & Pardee, A. B. (1956). *Biochim. biophys. Acta*, **19**, 236.
Laskowski, M. (1959). *Ann. N.Y. Acad. Sci.* **81**, 776.
Potter, J. L. & Laskowski, M. (1959). *J. biol. Chem.* **234**, 1263.
Zahn, R. K. (1959). *Nature, Lond.*, **184**, 1324.
Zahn, R. K. & Kleinschmidt, A. (1959). *Klin. Wschr.* **37**, 1153.
Zahn, R. K., Müller, H. K., Müller, S. & Zahn, G. (1959). *Biochem. Z.* **331**, 518.
Zahn, R. K., Rajewsky, K., Kiefer, R. G., Eitel, L. & Zahn, G. (1959). *Naturwissenschaften*, **46**, 518.

(46) Soluble Nucleotides of Baker's Yeast.

By J. K. MIETTINEN and T. SAVIOJA. (*Biochemical Institute, Helsinki, Finland*)

Low-molecular nucleotides of growing baker's yeast, labelled with ^{32}P by incubation for 20 min. under aerobic conditions, were extracted with cold 8% trichloroacetic acid. After removal of the trichloroacetic acid by ether extraction the solution was analysed by paper chromatography and by chromatography on Dowex-1 using formic acid and ammonium formate as eluants. The ultraviolet absorption of the eluate at 260 mμ and its radioactivity were continuously recorded. About 20 ultraviolet-absorbing peaks were registered. The corresponding fractions were purified by absorption on Norit charcoal and analysed by two-dimensional paper chromatography for phosphate esters and possible ninhydrin-positive compounds, and, after mild or strong hydrolysis, for the purine or pyrimidine bases, sugars and amino acids thus liberated. Diphosphopyridine nucleotide, triphosphopyridine

nucleotide and the 5-mono-, di-, and tri-phosphates of adenosine, guanosine and cytidine were identified. Uridine derivatives showed a tendency to be retained irreversibly by Norit but the 5′-mono-, di-, and tri-phosphates and several other not-yet-fully characterized uridine derivatives were identified by paper chromatography of the original extract. Several further unknown nucleotides, which have been only partially characterized, were also isolated from the eluate of Dowex chromatography. All other nucleotides except cytidine monophosphate, guanosine monophosphate and triphosphopyridine nucleotide became labelled during the 20-min. incubation.

Only the diphosphopyridine nucleotide fractions contained small amounts of two ninhydrin-positive compounds of a peptide nature. From the other fractions only traces of glycine and glutamic acid—less than one-tenth of the nucleotide in the fraction on a molar basis—were liberated during strong hydrolysis.

The amount of possible nucleotide–peptide complexes of a mixed anhydride type was determined quantitatively in the original extract by the hydroxylamine–$FeCl_3$ reaction. The extract did not contain such compounds, except in a very small amount compared with the free nucleotides, i.e. less than one-fiftieth on a molar basis. Even though such compounds have been shown in water macerates of baker's yeast (Konigsberger, Van der Grinten & Overbeek, 1957), and have been isolated from ethanol extracts of brewer's yeast (Harris, Davies & Parsons, 1958; Harris & Davies, 1959), their amount in growing yeast seems to be so low that it does not seem very probable that they could play a general role in protein synthesis in yeast.

REFERENCES

Harris, G. & Davies, J. V. (1959). *Nature, Lond.*, **184**, 788.
Harris, G., Davies, J. V. & Parsons, R. (1958). *Nature, Lond.*, **182**, 1565.
Koningsberger, V. V., Van der Grinten, C. O. & Overbeek, J. T. G. (1957). *Biochim. biophys. Acta*, **26**, 483.

(47) Glucose Breakdown in Chick-Embryo Cells Infected with Influenza Virus.

By H. G. Klemperer. (*National Institute for Medical Research, London, N.W. 7*)

The growth of virus in many tissue culture systems is known to be dependent on the presence of added substrate. The onset of changes in the rate of glucose breakdown has been correlated with the appearance of influenza virus components in infected tissue culture cells.

Within 1·5 hr. of infection, when the complement-fixing antigen first appeared, the rate of aerobic lactate formation was increased 50–100 % without any change in oxygen uptake. Aerobic glycolysis by control cells was stimulated approximately 100 % by 0·01 M-inorganic phosphate and 200 % by 0·01 M-phosphate plus 0·003 M-adenosine monophosphate. Compared with control cells, aerobic lactate formation by infected cells was 50 % greater in the presence of phosphate but about the same in the presence of phosphate plus adenosine monophosphate, suggesting that glycolysis is stimulated in the infected cell by an increased supply of phosphate acceptor.

Experiments with [1-^{14}C]- and [6-^{14}C]-glucose indicated that less than 10 % of the glucose taken up by control cells was directly oxidized to pentose phosphate and that this pathway was not an important source of ribose for ribonucleic acid synthesis in either control or infected cells. However, within 3 hr. of infection, when the viral haemagglutinin appeared, the rate of direct oxidation of glucose increased by 30–60 %. There was no increased dehydrogenase activity for glucose 6-phosphate or 6-phosphogluconate in infected cells. Methylene blue (0·5 mM) greatly increased the rate of direct oxidation of glucose, indicating that glucose breakdown via this pathway was limited by the rate of regeneration of triphosphopyridine nucleotide from the reduced coenzyme.

The possible relationship of these changes to virus synthesis will be discussed.

(48) Restriction of the Availability of Particle-bound Nucleotides as a Controlling Mechanism of Glucose Metabolism

By R. Balázs and D. Richter. (*Neuropsychiatric Research Unit, Medical Research Council, Woodmansterne Road, Carshalton, Surrey*)

When mitochondrial fractions from rat brain are incubated in a medium containing glucose and pyruvate without added cofactors ('unfortified' system; Balázs, 1959), glucose consumption and lactate formation are both inhibited under aerobic conditions by about 80 %.

(A) The aerobic inhibition of glucose consumption is completely removed by adding adenosine triphosphate (ATP), and it is due apparently to a restricted availability of mitochondrial ATP for hexokinase under aerobic conditions. The reduced availability of mitochondrial ATP is not due to increased leakage of ATP from the particles under aerobic conditions since the aerobic inhibition was completely reversed by changing the gas phase and measurements of the nucleotide content (acid-extractable material absorbing at 260 mμ) of the mitochondria showed that this is similar under aerobic and anaerobic conditions.

Another possibility was that the formation of ATP from adenosine diphosphate (ADP) was

reduced under aerobic conditions. Since glycolysis is inhibited also at other points below the hexosed diphosphate step under aerobic conditions (Balázs, 1959), ATP formation might be reduced if oxidative phosphorylation was inactive in this 'unfortified' system. It could be shown, however, that respiration was tightly coupled and hence ADP phosphorylated to ATP (Hess & Chance, 1959), since the respiration rate was increased 2–4 times by adding ADP or uncouplers of phosphorylation (dinitrophenol; dicoumarol).

These experiments give evidence that the mitochondrial ATP is bound in a form in which its accessibility for hexokinase is reversibly restricted under aerobic conditions. Anaerobic conditions, and apparently respiratory inhibitors and uncouplers abolish this 'compartmentation' of the mitochondrial ATP. The possible role of compartmentation in the control of glucose metabolism has been discussed by Lynen & Koenigsberger and others (Racker & Wu, 1959; Chance, 1959).

(B) Although addition of ATP abolished the aerobic inhibition of glucose phosphorylation in this system, lactate formation remained inhibited by about 70 %. In a system containing hexose diphosphate, glucose, pyruvate, oxoglutarate, diphosphopyridine nucleotide and ADP, the aerobic inhibition of lactate formation was partly removed by adding dinitrophenol and partly by crystalline triose phosphate dehydrogenase; it was completely removed by adding both. It is concluded that the aerobic inhibition of lactate formation is mediated partly by limited availability of nucleotide cofactors and partly by a specific inhibition of triose phosphate dehydrogenase (Balázs, 1959).

REFERENCES

Balázs, R. (1959). *Biochem. J.* **72**, 561.
Chance, B. (1959). *Ciba Foundation Symposium on Regulation of Cell Metabolism*, p. 91. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: Churchill.
Hess, B. & Chance, B. (1959). *J. biol. Chem.* **234**, 3031.
Lynen, F. & Koenigsberger, R. (1951). *Ann. Chem., Justus Liebigs*, **573**, 60.
Racker, E. & Wu, R. (1959). *Ciba Foundation Symp. on Regulation of Cell Metabolism*, p. 205. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. & A. Churchill Ltd.

(49) Promotion of Growth and Amino Acid Incorporation into Protein of Rats by a Non-diabetogenic Derivative of Alloxan.

By K. L. Manchester and F. G. Young. (*Department of Biochemistry, University of Cambridge*)

Manchester & Young (1960) found that although the induction of alloxan-diabetes ultimately depresses the rate of incorporation of [^{14}C]glycine into the protein of isolated rat diaphragm, the same tissue taken from rats 48 hr. after the administration of alloxan shows a substantially elevated level of incorporation of amino acid into protein. This action of alloxan was not found to be dependent on the presence of the pituitary gland, for the effect was observable in diaphragm from hypophysectomized rats, but was dependent on the presence of the adrenals and not observed with diaphragm from adrenalectomized rats.

The question arises as to whether this stimulation of amino acid incorporation 48 hr. after the administration of alloxan is a result of the onset of diabetes or represents an action of alloxan different from its diabetogenic activity. Brückmann & Wertheimer (1947) found that a substituted derivative of alloxan, 1-phenylalloxan, possesses no observable diabetogenic or toxic activity. Administration of this compound in a dose equimolar with that of alloxan used to induce diabetes (200 mg. of alloxan/kg., subcutaneously) has been found to have if anything a greater effect than alloxan in elevating the rate of amino acid incorporation by diaphragm removed from rats 48 hr. after its administration, although the blood-sugar level at this time is normal. This substance appears to be without activity in the adrenalectomized rat or the adrenalectomized rat treated with cortisol. Daily administration of phenylalloxan to female rats of about 200 g. for a period of 7 days has been found to result in some elevation of body weight during the period of treatment and the following 10-day period. This extra weight is not subsequently lost.

The results confirm the suggestion of Manchester & Young (1960) that alloxan and its derivatives, irrespective of their diabetogenic potency, may stimulate the release by the adrenal gland of some growth-promoting agent, possibly of androgenic nature, which can promote both amino acid incorporation by diaphragm and an increase of body weight.

REFERENCES

Brückmann, G. & Wertheimer, E. (1947). *J. biol. Chem.* **168**, 241.
Manchester, K. L. & Young, F. G. (1960). *Biochem. J.* **75**, 487.

(50) Insulin-stimulated Incorporation of [^{14}C]Glucose into Two Glucose Complexes of Rat Diaphragm.

By O. Walaas, E. Walaas, B. Borrebæk and T. Kristiansen (introduced by P. N. Campbell). (*Institute of Medical Biochemistry and Physiology, Department of Biochemistry, University of Oslo, Norway*)

It has recently been reported that [^{14}C]glucose is rapidly distributed into a peptide com-

plex of rat diaphragm *in vitro* (Walaas, Borrebæk, Kristiansen & Walaas, 1960). Similar results have been obtained by incubation of bundles of rat-leg adductor muscles in a medium containing [^{14}C]-glucose. This shows that the glucose complexes, compound A and compound B, are constituents of the muscle tissue. In experiments on rat diaphragm the effect of insulin on the incorporation of [^{14}C]glucose into compound A and into compound B have been studied. Incubation was performed in Krebs–Ringer phosphate medium containing 140 mg. of glucose/100 ml. at 30°. [U-^{14}C]-Glucose (10 μc) was added and diluted to a specific activity of 3·6 μc/mg. Four pairs of hemidiaphragms without and with addition of 0·1 unit of insulin/ml. were incubated in each experiment. When incubation was finished, the diaphragms were extracted with 85 % ethanol. Compound A and compound B both remained in solution, and were purified by paper chromatography and paper electrophoresis. The radioactivity was detected and quantitatively estimated with a paper-strip scanner. In short-time incubation experiments insulin stimulates incorporation of [^{14}C]glucose into compound A as well as into compound B. The insulin effect on compound A appears after an incubation period of 5 min. and reaches a maximum within 7 min. An effect of the order of 200 % has been observed. The effect by insulin on compound B is less pronounced. However, the insulin stimulation slightly exceeds 50 % on incubation for 10 min. Some evidence has been obtained that the glucose complexes may be involved in the insulin-sensitive membrane-transport mechanism for glucose.

REFERENCE

Walaas, O., Borrebæk, B., Kristiansen, T. & Walaas, E. (1960). *Biochim. biophys. Acta*, (in the Press).

(51) Amylo-1:6-Glucosidase Activity in Tissues of Children with Glycogen-storage Disease.

By H. G. Hers.* (*Laboratory of Physiological Chemistry, University of Louvain, Louvain, Belgium*)

The absence of amylo-1:6-glucosidase in the muscles of two children with a generalized form of glycogen-storage disease was first observed by Illingworth, Cori & Cori (1956). The activity of the enzyme in the liver was not determined by these authors, because the method used at that time could not be applied to hepatic tissue. Using a new method, based on the incorporation of [^{14}C]glucose in glycogen (Hers, 1959), we have noted a complete absence of the enzyme in the liver in nine out of thirty-six cases of glycogen-storage disease. Muscle biopsies were obtained in six of these cases. In five of them, the enzymic deficiency of the liver was shared by the muscles, which also showed abnormal glycogen deposition. In one, however, the muscle-glycogen level did not exceed 1·2 % and amylo-1:6-glucosidase activity, though slightly lower than normal, could easily be measured.

The nine cases were distributed nearly equally among both sexes and many of them had relatives similarly affected or had parents who were first cousins.

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REFERENCES

Illingworth, B., Cori, G. T. & Cori, C. F. (1956). *J. biol. Chem.* **218**, 123.
Hers, H. G. (1959). *Rev. intern. Hépatol.* **9**, 35.

(52) Reduction in Oxidative Activities of Some Bacteria during Inhibition of Growth by Streptomycin.

By R. Hancock. (*Department of Bacteriology, University of Manchester*)

During the period in which the growth of exponential-phase cultures of *Staphylococcus aureus* (strain Duncan) becomes inhibited by streptomycin, changes occur in the distribution of ^{14}C from [^{14}C]-glucose, accompanied by a progressive decrease in the rate of glucose oxidation by harvested organisms (Hancock, 1960). Such organisms also show reduced rates of oxidation (6–55 % of control values) for oxidation of lactate, formate and glycerol. This appears to be a specific effect of streptomycin and not a consequence of cessation of growth. Cells of another strain of *S. aureus* show reduced rates of succinate and glucose oxidation after growth in the presence of streptomycin (Jackson, 1958).

Similar changes have now been found in cultures of *Escherichia coli* (strain B) and *Bacillus megaterium* (strain KM) during inhibition of growth by streptomycin. As with *S. aureus* (Hancock, 1960), streptomycin is not bactericidal to these organisms in the absence of growth. In cultures of *E. coli*, the rates of oxidation of glucose, lactate, succinate and pyruvate by organisms growing in CG medium (McQuillen & Roberts, 1954) in the presence of streptomycin are progressively reduced, and, when growth is completely inhibited, reach 20–40 % of those of normal organisms. At this time, hot-water extracts of normal and streptomycin-inhibited organisms contain similar quantities of amino acids and substances absorbing light at 260 mμ. No differences have been observed in the stability, or the internal osmotic pressure (determined by the method of Mitchell & Moyle, 1957), of 'protoplasts',

prepared according to Mahler & Fraser (1956), from streptomycin-inhibited and normal organisms. It therefore seems unlikely that the reduced oxidative activities are caused by breakdown of the permeability barrier, which has been suggested (Anand & Davis, 1960) as an early effect of streptomycin on *E. coli* strain W.

Organisms harvested from cultures of *B. megaterium* during inhibition of growth by streptomycin show rates of oxidation of all substances tested which are also progressively reduced, and finally reach 8–40% of those of normal organisms. Streptomycin-inhibited organisms give stable protoplasts with an internal osmotic pressure slightly (about 10%) higher than normal protoplasts. It appears that in this organism also, the protoplast membrane is intact when growth inhibition is complete, although oxidation reactions are impaired.

REFERENCES

Anand, N. & Davis, B. D. (1960). *Nature, Lond.*, **185**, 22.
Hancock, R. (1960). *Biochem. J.* **74**, 12P.
Jackson, F. L. (1958). *Nature, Lond.*, **181**, 281.
McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
Mahler, H. R. & Fraser, D. (1956). *Biochim. biophys. Acta*, **22**, 197.
Mitchell, P. & Moyle, J. (1957). *J. gen. Microbiol.* **16**, 184.

(53) The Nature of the Diauxic Effect with Glucose and Organic Acids in *Pseudomonas aeruginosa*.

By W. A. Hamilton and E. A. Dawes.
(*Department of Biochemistry, University of Glasgow*)

We have shown previously that during diauxic growth of *Pseudomonas aeruginosa* in mineral salt media containing glucose and an organic acid, preferential utilization of the acid occurs (Hamilton & Dawes, 1959); this is in contrast to previously recorded cases with *Aerobacter aerogenes*.

Although the tricarboxylic acid cycle is constitutive in *P. aeruginosa*, it has been shown that entry of various exogenous intermediates of the cycle into glucose-grown cells is mediated by inducible permeases (Clarke & Meadow, 1959). Consequently three possible explanations of the diauxic effect suggest themselves, namely (1) the enzymes of glucose metabolism are constitutive but entry of glucose is effected by a permease system, the formation and/or operation of which is inhibited by organic acids; (2) some or all of the glucose-metabolizing enzymes are inducible and their formation and/or functioning are inhibited by the simultaneous presence of an organic acid, but the permease is constitutive, and (3) both the permease and system of glucose enzymes are inducible.

P. aeruginosa possesses enzymes for the direct oxidation of glucose to gluconic and 2-oxogluconic acids (Stokes & Campbell, 1951; Claridge & Werkman, 1953), and since isotopic studies suggest the operation of the Entner–Doudoroff (1952) pathway and also the preferential elimination of C-1 of glucose (Wang, Stern & Gilmour, 1959), we have assayed some of the enzymes characteristic of these pathways of metabolism in extracts of glucose-grown cells. Enzymes found present included glucose and gluconic dehydrogenases, hexokinase, gluconokinase, 2-oxogluconokinase, glucose 6-phosphate and 6-phosphogluconate dehydrogenases, and the combined action of the dehydrase and aldolase of the Entner–Doudoroff route was demonstrated. Cells grown on organic acids, however, contained extremely low basal levels of these enzymes, incubation with glucose producing a very marked increase in enzyme activity. Studies using chloramphenicol with whole cells indicated that this adaptation involved net synthesis rather than activation of pre-existing enzymes. Activity of acid phosphatases was negligible and uninfluenced by growth substrate.

Methyl α-glucoside is a non-metabolized inducer of glucose oxidation but no evidence was obtained for a mechanism for its concentration within the cell. Other results, however, suggest the existence of a permease for glucose.

These results were discussed in terms of current theories of enzyme induction and repression.

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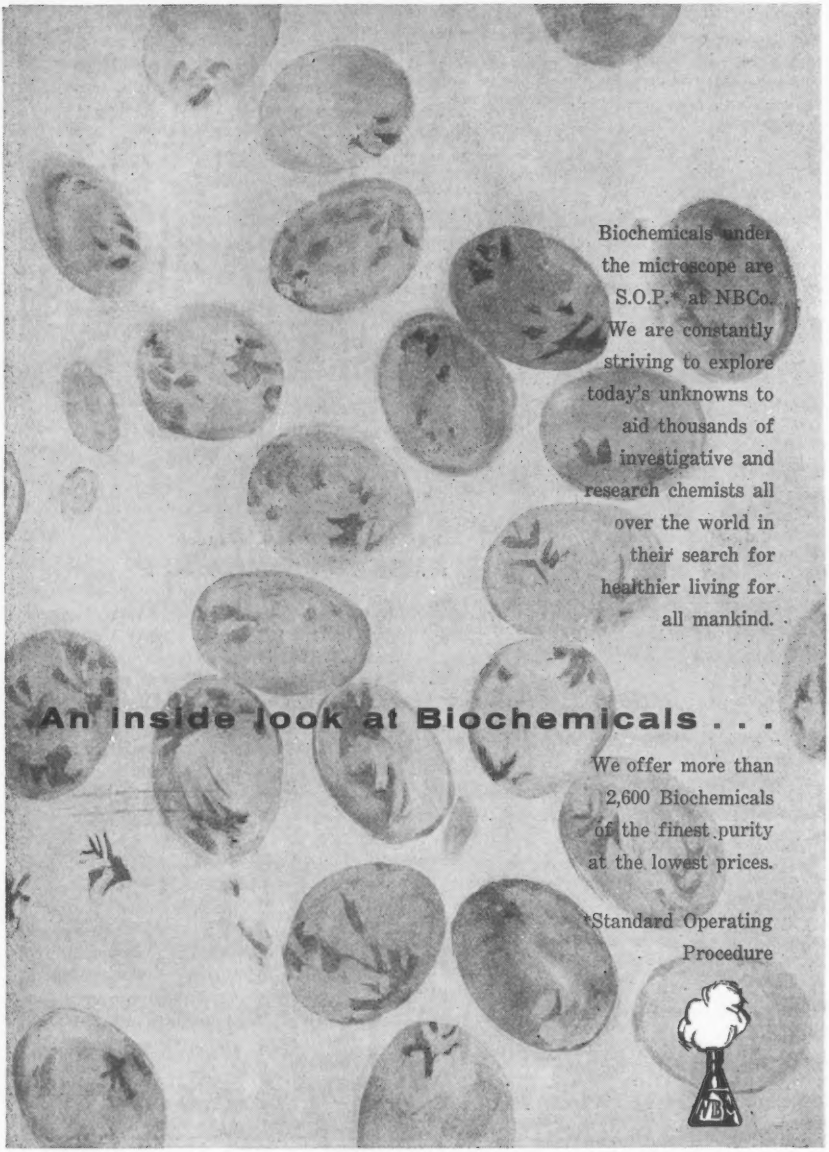
REFERENCES

Claridge, C. A. & Werkman, C. H. (1953). *Arch. Biochem. Biophys.* **47**, 99.
Clarke, P. H. & Meadow, P. M. (1959). *J. gen. Microbiol.* **20**, 144.
Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
Hamilton, W. A. & Dawes, E. A. (1959). *Biochem. J.* **71**, 25P.
Stokes, F. N. & Campbell, J. J. R. (1951). *Arch. Biochem. Biophys.* **30**, 121.
Wang, C. H., Stern, I. J. & Gilmour, C. M. (1959). *Arch. Biochem. Biophys.* **81**, 489.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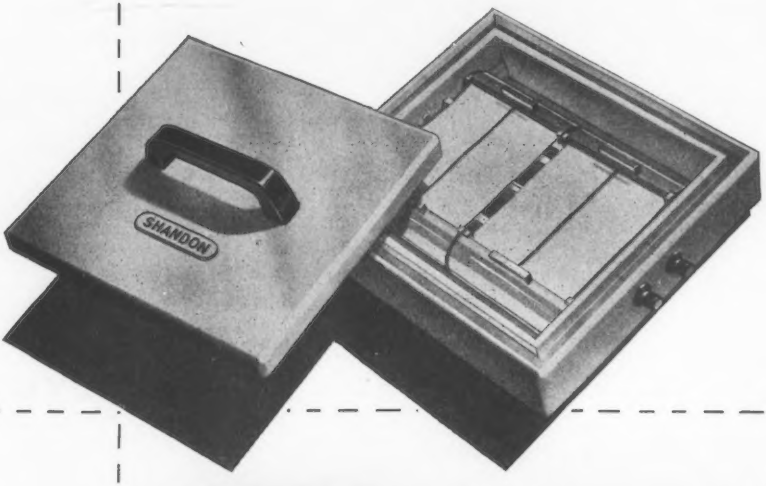

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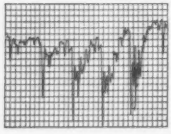

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